

Mutations in exon 14 of dihydropyrimidine dehydrogenase and 5-Fluorouracil toxicity in Portuguese colorectal cancer patients

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Purpose: Dihydropyrimidine dehydrogenase is a critical enzyme in the catabolism of 5-Fluorouracil, a drug frequently used in cancer therapy. Patients with deficient dihydropyrimidine dehydrogenase activity are at risk of developing severe 5-Fluorouracil-associated toxicity. Genetic analysis of the gene coding for dihydropyrimidine dehydrogenase has shown that mutations in exon 14, especially the splice-site mutation IVS14+1G→A, were associated with dihydropyrimidine dehydrogenase enzymatic deficiency. **Methods:** We evaluated the frequency of mutations in exon 14 of dihydropyrimidine dehydrogenase (*DPYD*) gene in 73 unselected colorectal cancer patients treated with 5-Fluorouracil after surgery at a Portuguese Cancer Institute. **Results:** Sequencing the entire exon 14 allowed the detection of mutations in two of the 73 patients (2.7%), namely two of the eight (25%) patients who presented grade 3-4 toxicity after 5-Fluorouracil chemotherapy. One patient was heterozygous for the splice-site mutation IVS14+1G→A, whereas the second patient was heterozygous for a novel missense mutation 1845G→T (E615D) in exon 14 of *DPYD* gene. **Conclusion:** We conclude that mutations in exon 14 of *DPYD* gene are responsible for a significant proportion of life-threatening toxicity to 5-Fluorouracil, and should therefore be excluded before its administration to cancer patients. *Genet Med* 2004;6(2):102–107.

Key Words: *DPYD mutations, dihydropyrimidine dehydrogenase, 5-FU toxicity, 5-FU catabolism, chemotherapy*

5-Fluorouracil (5-FU) is one of the most commonly used chemotherapeutic drugs for the treatment of malignant neoplasms, namely colorectal cancer. 5-FU is a pyrimidine analogue, giving rise to cytotoxic metabolites after metabolization by the pyrimidine pathways. One of them, the 5-fluoro-dUMP, inhibits thymidylate synthase, thus impairing DNA synthesis. Additional cytotoxic effects arise from 5-FU metabolites being incorporated into RNA and DNA.¹

Dihydropyrimidine dehydrogenase (DPD) is the initial and rate-limiting enzyme in the catabolism of 5-FU. It has been reported that more than 80% of the administered 5-FU is catabolized by DPD,^{2–4} so the activity of this enzyme may be of paramount importance to predict the efficacy and toxicity of this drug. Deficiency in DPD enzyme activity has been correlated with considerable delay in 5-FU clearance from plasma.^{4,5} The important role of DPD in 5-FU-based chemotherapy has been demonstrated in cancer patients with major deficiency of this enzyme's activity. These patients may present severe tox-

icity after the administration of 5-FU, including diarrhea, neutropenia, and neurotoxicity, and death may occasionally occur.^{6–9} Population studies suggested that, although total deficiency is rare, as many as 3% to 5% of the population may have low enzyme levels and thus be at increased risk of severe toxicity if treated with 5-FU.¹⁰

Several of these patients are genotypically heterozygous for a mutant *DPYD* allele.^{11–16} To date, more than 30 variant *DPYD* alleles have been identified, some of which result in decreased activity of DPD enzyme.^{17–19} Analysis of the prevalence of the various mutations has shown that the splice-site mutation IVS14+1G→A is by far the most common.^{13,14} This mutation results in a 165-bp deletion (corresponding to exon 14) in the *DPYD* mRNA. The resulting protein product is truncated by 55 amino acids, and its catalytic activity is virtually absent. Some reports have shown that the splice-site mutation IVS14+1G→A is present in approximately 1% of the Caucasian population.^{17,18} In addition, a recent study showed that approximately 50% of the patients who had experienced severe 5-FU toxicity (grade 3-4) were heterozygous for this exon-skipping mutation.¹⁹

To evaluate the frequency of mutations in exon 14 of *DPYD* and its contribution to 5-FU toxicity in Portuguese colorectal cancer (CRC) patients treated with 5-FU, we have performed full sequencing of exon 14 in 73 CRC patients treated with this adjuvant chemotherapeutic regimen after surgery.

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Table 1
Clinical data and treatment modalities of 73 patients with colorectal adenocarcinoma

Patient no.	Sex	Age	Tumor location	Disease stage	Treatment	
1	F	57	Rectal	1	5-FU-LV	Bolus
2	F	54	Rectal	2	5-FU-FA	Bolus
3	F	48	Rectal	2	5-FU-LV	Bolus
4	M	56	Rectal	2	5-FU-FA	Bolus
5	F	68	Rectal	2	5-FU-FA	Bolus
6	F	71	Sigmoid	2	5-FU-FA	Bolus
7	M	65	Transverse colon	3	5-FU-FA	Bolus
8	M	61	Right colon	3	5-FU-LV	Bolus
9	F	51	Rectal	3	5-FU-LV	Bolus
10	F	64	Transverse colon	3	5-FU-FA	Bolus
11	M	67	Rectal	2	5-FU-FA	Bolus
12	M	68	Right colon	2	5-FU-FA	Bolus
13	F	68	Left colon	2	5-FU-FA	Bolus
14	M	67	Rectal	1	5-FU-LV	Bolus
15	M	43	Left colon	2	5-FU-LV	Bolus
16	F	59	Rectal	4	5-FU-FA	Bolus
17	M	67	Rectal	2	5-FU-LV	Bolus
18	F	58	Rectal	2	5-FU-FA	Bolus
19	F	64	Rectal	3	5-FU-FA	Bolus
20	F	65	Rectal	4	5-FU-LV	Bolus
21	M	49	Rectal	4	5-FU-FA	Bolus
22	F	37	Rectal	4	5-FU-RT/5-FU-FA	Bolus
23	F	68	Rectal	3	5-FU-FA	Bolus
24	M	78	Left colon	2	5-FU-FA	Bolus
25	F	41	Sigmoid	3	5-FU-FA	Bolus
26	M	49	Left colon	2	5-FU-LV	Bolus
27	F	63	Sigmoid	2	5-FU-FA-CPT11	IC
28	M	62	Rectal	4	5-FU-FA-CPT11	IC
29	M	61	Rectal	3	5-FU-FA	Bolus
30	M	65	Rectal	3	5-FU-FA/5-FU-FA-CPT11	Bolus/IC
31	F	65	Sigmoid	3	5-FU-FA	Bolus
32	F	57	Sigmoid	2	5-FU-FA/5-FU/5-FU-FA-CPT11	Bolus/Bolus/IC
33	F	36	Rectal	3	5-FU-CPT11-Oxal	IC
34	F	85	Sigmoid	2	5-FU-FA	Bolus
35	M	80	Right colon	3	5-FU-FA	Bolus
36	M	55	Left colon	3	5-FU-FA/5-FU-CCNU-Oncovim	Bolus
37	F	49	Rectal	3	5-FU-FA	Bolus
38	M	64	Rectal	2	5-FU-RT/5-FU-FA/5-FU-FA-CPT11	Bolus/Bolus/IC
39	M	40	Rectal	2	5-FU-RT	Bolus
40	M	41	Rectal	2	5-FU-RT	IC
41	M	57	Rectal	3	5-FU-FA	Bolus

—Continued

Table 1
Continued

Patient no.	Sex	Age	Tumor location	Disease stage	Treatment	
42	F	69	Rectal	2	5-FU-FA/5-FU-FA-CPT11	Bolus/IC
43	F	65	Rectal	2	5-FU-FA	Bolus
44	F	74	Rectal	2	5-FU-FA/5-FU-FA-CPT11	IC/IC
45	M	70	Rectal	2	5-FU-RT	IC
46	M	49	Rectal	3	5-FU-FA	Bolus
47	M	44	Rectal	3	5-FU-RT	IC
48	M	31	Sigmoid	2	5-FU-FA	IC
49	F	46	Sigmoid	4	5-FU-RT/5-FU-FA	IC
50	F	48	Rectal	4	5-FU-FA-CPT 11	IC
51	F	50	Right colon	3	5-FU-Heparin	Bolus
52	M	37	Rectal	3	5-FU-RT	IC
53	M	72	Rectal	3	5-FU-FA-RT	IC
54	M	45	Rectal	3	5-FU-FA	Bolus
55	F	48	Rectal	3	5-FU-FA-RT	Bolus
56	F	38	Rectal	3	5-FU-FA-RT	IC
57	F	61	Rectal	3	5-FU-RT	IC
58	F	50	Rectal	3	5-FU-FA	Bolus
59	F	41	Rectal	2	5-FU-RT	IC
60	M	71	Rectal	3	5-FU-RT	IC
61	F	31	Rectal	3	5-FU-FA	IC
62	F	40	Rectal	3	5-FU-RT/5-FU-FA	IC
63	M	61	Rectal	3	5-FU-FA	IC
64	F	67	Rectal	3	5-FU-RT/5-FU-FA	IC
65	F	53	Rectal	3	5-FU-RT	IC
66	F	66	Rectal	2	5-FU-RT	Bolus
67	M	47	Rectal	3	5-FU-RT	IC
68	M	72	Sigmoid	3	5-FU-FA-Oxal	IC
69	M	68	Rectal	3	5-FU-FA	IC
70	M	52	Rectal	3	5-FU-RT	IC
71	M	68	Rectal	4	5-FU-RT/5-FU-FA	IC/IC
72	F	46	Sigmoid	3	5-FU-FA	Bolus
73	F	74	Right colon	2	5-FU-Heparin	Bolus

5-FU, 5-Fluorouracil; FA, folinic acid; LV, levamisole; RT, radiotherapy; Oxal, oxaliplatin; IC, continuous infusion.

MATERIALS AND METHODS

Patients and toxicity evaluation

Seventy-three consecutive patients [34 males and 39 females; mean age, 59 (range, 31–85) years] with CRC, who were treated with potentially curative surgical resections followed by adjuvant 5-FU–based chemotherapy, were included in this study (Table 1). Informed consent was obtained from all patients studied. Chemotherapy toxicity was evaluated according to the National Cancer Institute toxicity scale.²⁰ The main doc-

umented toxicities were hematological, gastrointestinal, mucocutaneous, and neurological.

Mutation analysis

Genomic DNA was isolated from peripheral mononuclear cells using a DNA Extraction Kit (Invitrogen) following the manufacturer's instructions. PCR amplification of exon 14 and its flanking intronic regions was performed using the following primer set¹⁶: DPD14F, 5'-GTAAGAAGGACCTCATA-

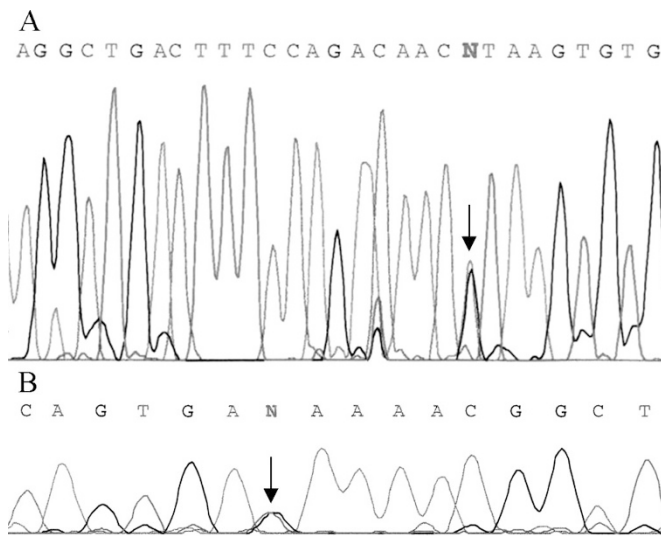


Fig. 1. Sequence analysis of exon 14 of *DPYD*. A, Patient 69 was shown to be heterozygous for the splice-site mutation IVS14+1G→A. B, Patient 22 was shown to be heterozygous for the missense mutation 1845G→T (E615D).

AAATATTGTC-3'; DPD14R, 5'-GAATTGGATGTTTA-AATAAACATTCACCAAC-3'. Amplification of exon 14 was performed in a 25 μ L reaction mixture containing 10 mmol/L Tris-HCL (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L MgCl₂, 10 pmol of each primer, 250 μ mol/L of each deoxynucleotide triphosphate, 0.2 Units of Taq polymerase, and 50 ng of genomic DNA. After initial denaturation for 2 minutes at 95°C, amplification was performed for 35 cycles (1 minute 95°C, 1 minute 55°C, 1 minute 72°C). The expected fragment size with this primer set is 342 bp. The PCR products were separated on 2% agarose gels, stained with ethidium bromide, purified using a GFX PCR DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech) and used for direct sequencing. Sequence analysis was performed on an Applied Biosystems model 310 automated DNA sequencer using the dye-terminator method.²¹

Statistical analysis

Comparisons of allelic frequencies between patients with and without grade 3-4 5-FU toxicity was performed with Fisher exact test. Two-tailed values of $P < 0.05$ were considered statistically significant.

RESULTS

Identification of mutations in exon 14 of *DPYD*

Analysis of exon 14 of the *DPYD* gene in 73 patients with CRC revealed the presence of mutations in two of them (2.7%). One patient was heterozygous for the splice-site mutation IVS14+1G→A and one patient was heterozygous for a novel missense mutation 1845G→T (E615D) in exon 14 of *DPYD* (Fig. 1). Therefore, the prevalence of each of these mutant alleles in colorectal patients was 0.7%.

Clinical evaluation of toxicity

Of the 73 patients, 65 presented grade 1-2 toxicity and eight (11%) suffered from grade 3 or 4 toxicity related to 5-FU chemotherapy (Table 2). The two mutations described above were found in two of the eight patients (25%) who developed grade 3 or 4 toxicity. A statistically significant association between exon 14 *DPYD* gene mutations and grade 3-4 5-FU toxicity was found ($P = 0.01$). The patient heterozygous for the splice-site mutation IVS14+1G→A was a man with a rectal carcinoma diagnosed at age 64 (pT3N1/10M0). He was submitted to anterior resection of the upper rectum and treated postoperatively with radiotherapy, followed by adjuvant chemotherapy with 5-FU-folinic acid (bolus). Therapy was stopped after the second cycle due to grade 4 febrile neutropenia and grade 3 digestive toxicity. The patient heterozygous for the missense mutation 1845G→T was a woman with a rectal cancer diagnosed at age 37 (pT4N2M0). She was submitted to abdominoperineal resection of the lower third of the rectum. Radiotherapy with 5-FU chemotherapy sensibilization was started after surgery. After radiotherapy was completed, 5-FU folinic acid chemotherapy was administered (bolus). On the 6th day after chemotherapy, grade 4 mucositis was documented, followed by febrile pancytopenia and sepsis due to *Pneumocystis carinii*. Mechanical ventilation support was necessary due to respiratory failure.

Sensitivity and specificity of *DPYD* mutations

Because two of the eight patients with grade 3-4 toxicity were shown to have *DPYD* mutations and all patients with grade 0-2 toxicity were negative, the sensitivity and specificity of the test is 25% and 100%, respectively.

Positive and negative predictive value

The positive predictive value of the test was 100% (2/2), whereas its negative predictive value was 92% (65/71).

DISCUSSION

Several population studies have demonstrated that total DPD deficiency is rare, but as many as 3% to 5% of the population may have low enzyme levels and thus be at increased risk for severe toxicity if treated with 5-FU.^{10,22} It has subsequently been demonstrated that a number of these individuals were genotypically heterozygous for a mutant *DPYD* allele,¹¹⁻¹⁶ but data are scant on the frequency of mutations in *DPYD* among cancer patients. In this study, we showed that the prevalence of the mutation in exon 14 of *DPYD* in a CRC population treated with 5-FU was 2.7%. Exon 14 *DPYD* mutations showed a statistically significant association with grade 3-4 5-FU toxicity ($P = 0.01$). The frequency of *DPYD* in our colorectal cancer population was not significantly different from that reported by Ridge et al.¹⁵ (2.7 vs. 4%).

To date, more than 30 variant *DPYD* alleles have been identified, some of which clearly result in decreased DPD activity.¹⁷⁻¹⁹ In our group of patients, two different mutations were

Table 2

Toxicity profile and exon 14 *DPYD* mutations in the eight patients (11% of the 73 selected) suffering from severe 5-FU–related toxicity (National Cancer Institute toxicity scale)

Patient no. (sex, age)	5-FU–related toxicity									
	Hematological grade			Gastrointestinal grade		Mucositis grade	Dermatological grade	Neurological grade	Other	Mutation
	Neutropenia	Thrombocytopenia	Anemia	Nausea/vomiting	Diarrhea					
14 (M, 68)	1	0	1	2	4	0	0	0		
22 (F, 37)	4	4	4	2	2	3	1	0	Infection-4 Pulmonary-4	1845G→T
26 (M, 49)	3	0	0	0	0	0	0	0		
42 (F, 69)	3	0	2	1	2	1	0	0		
55 (F, 48)	4	0	1	1	1	0	0	0		
58 (F, 50)	4	0	1	1	1	1	0	0		
68 (M, 72)	0	1	0	0	0	3	2	0		
69 (M, 68)	4	0	2	0	2	0	0	0	Fever-3 Infection-2	IVS14+1G→A

identified: the splice-site mutation IVS14+1G→A and the missense mutation 1845G→T. The mutation IVS14+1G→A is the most frequently detected in patients with complete DPD activity deficiency.^{13,14} This point mutation in the invariant GT splice donor site leads to the skipping of exon 14 in the *DPYD* mRNA, which results in a truncated protein by 55 amino acids with virtually no catalytic activity. This mutation was detected in one of eight patients with severe 5-FU toxicity, which is in line with other published studies.^{7,8,13–16} A few reports have recently described the prevalence of the exon 14–skipping mutation in colorectal cancer and healthy populations. Van Kuilenburg et al.¹⁸ reported a frequency of 0.9% in 1357 Caucasian blood donors. Furthermore, Raida et al.¹⁷ reported the IVS14+1G→A mutation in 0.94% of the alleles in 851 Caucasian blood donors. The allelic frequency found in the present study (0.7%) does not significantly differ from the findings in these two reports.

We identified a novel missense mutation, 1845G→T, in exon 14 of *DPYD* in a patient who suffered from severe 5-FU toxicity. Although DPD activity was not determined in this patient, the observed toxicity profile and the location of the mutation in an important functional domain of the protein (a uracil-binding site), suggest that this alteration may be pathogenic. A recent study demonstrated that missense mutations present in highly conserved regions of the *DPYD* gene may interfere with protein function.²³ However, additional studies, such as determination of DPD activity and other functional studies, are necessary to determine the significance of this mutation.

Raida et al.¹⁷ has previously reported that 6 of 25 patients who experienced grade 3–4 toxicity upon 5-FU treatment carried the IVS14+1G→A mutation (five were heterozygous and one was homozygous for this splice-site mutation). In the present series, two of eight patients (25%) with grade 3–4 tox-

icity had mutations in exon 14 of *DPYD*, although only one (12.5%) was heterozygous for the IVS14+1G→A mutation. The fact that we detected a novel mutation (1845G→T) in exon 14 of *DPYD* indicates that full exon screening, not only the exon-skipping mutation, should be performed. Whereas our test revealed 100% positive predictive value and 100% specificity, six patients with severe toxicity to 5-FU treatment showed no mutations in exon 14 of *DPYD*, corresponding to a sensitivity of 25% and a negative predictive value of 92%. We cannot exclude the possibility that these patients are carriers of other loss-of-function mutations in the coding sequence or promoter region of *DPYD*.²⁴ Alternatively, increased expression of enzymes of the anabolic pathway may be responsible for the observed increased sensitivity to 5-FU in patients without *DPYD* mutations.

In conclusion, the common use of 5-FU in cancer therapy and the relatively high frequency of mutations in exon 14 of *DPYD* in patients developing grade 3–4 toxicity, indicate that genetic screening for the presence of *DPYD* mutations in cancer patients before the administration of 5-FU is appropriate, in order to prevent serious and sometimes lethal 5-FU-related toxicities.

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