Clinical relevance of different dihydropyrimidine dehydrogenase gene single nucleotide polymorphisms on 5-fluorouracil tolerance

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Abstract

Purpose: **Although single nucleotide polymorphisms (SNP) of the dihydropyrimidine dehydrogenase gene (*DPYD*) have been reported, which affect enzyme activity and the severity of 5-fluorouracil (5-FU) toxicity, no pretherapeutic detection has thus far been developed. We investigated 22 *DPYD* gene SNPs, their respective incidence, their link with grade 3 to 4 toxic side effects, and their management in practice: 9 were looked for in 487 patients, whereas 13 others were investigated in 171 patients. *Patients and Methods:* SNPs were detected before 5-FU-based treatment in WBC using a Pyrosequencing method. Close clinical and biological follow-up was done. *Results:* Five different SNPs were found in 187 patients (IVS14 + 1G>A, 2846A>T, 1679T>G, 85T>C, −1590T>C). Three hundred patients had no SNP. Forty-four patients had grade 3 to 4 toxic side effects in either the first or second cycle. Sixty percent of patients with either IVS14 + 1G>A or 2846A>T SNPs and the only patient with 1679T>G SNP experienced early grade 3 to 4 toxicity, compared with 0%, 5.5%, and 15% of those with either −1590T>C, 85T>C SNP, or no SNP, respectively. In cases with grade 3 to 4 toxicity, treatment either had to be quickly stopped, or could be safely continued with an individual dose adjustment. Sensitivity, specificity, and positive and negative predictive values of the detection of these three major SNPs as toxicity predictive factors were 0.31, 0.98, and 0.62 and 0.94, respectively.** ***Conclusion:*** **Pretreatment detection of three *DPYD* SNPs could help to avoid severe toxic side effects. This approach is suitable for clinical practice and should be compared or combined with pharmacologic approaches. In the case of dihydropyrimidine dehydrogenase deficiency, 5-FU administration often can be safely continued with an individual dose adjustment. [Mol Cancer Ther 2006;5(11):2895–904]**

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Introduction

5-Fluorouracil (5-FU), a widely used drug, can result in severe toxic side effects, including death, (1–3). More than 80% of the administered drug is catabolized by dihydropyrimidine dehydrogenase (DPD) in dihydrofluorouracil (4). DPD activity is subject to a wide variability, mainly due to genetic polymorphism. This results in a broad range of enzymatic deficiency from partial (3–5 % of population) to complete loss (0.2% of the population) of enzyme activity (5–7) and consequently to severe polyvisceral 5-FU-induced toxicity (8).

Considering the frequent use of 5-FU and oral fluoropyrimidines, pretreatment detection of this deficiency could prevent severe toxicity. At least 50% of patients who experienced highly toxic 5-FU effects were genotypically heterozygous or homozygous for known single nucleotide polymorphisms (SNP) in the *DPYD* gene (9, 10). In addition to the splice site variant IVS14 + 1G >A considered the most common SNP (52%), >30 variant *DPYD* alleles have been published, and it has been suggested that more than half might have a deleterious effect on DPD enzyme activity (9–11). The prevalence of patients who are heterozygous or homozygous for these relevant SNPs and then at risk of 5-FU-induced severe toxicity is about 3%, but, to date, the respective frequencies of the different SNPs and their effects on DPD activity in patients have not been adequately investigated (11, 12).

The purposes of the present prospective study were (*a*) to set up an accurate minisequencing method suitable for the detection of several SNPs in clinical practice; (*b*) to determine the clinically relevant *DPYD* gene SNPs and their respective frequencies, their effect on 5-FU pharmacokinetics, their and clinical tolerance; (*c*) to evaluate *DPYD* genotyping with pyrosequencing for detection of patients at risk of toxicity with 5-FU administration in a population of 487 cancer patients (13).

Patients and Methods

Patients and Treatment

This prospective study was conducted on 487 French Caucasian patients treated for advanced carcinomas or in

the adjuvant setting with 5-FU-based chemotherapy regimen. To be eligible, patients had to be naive of 5-FU, to have a WHO performance status <2, a life expectancy of at least 3 months, an age lower than 80 years, and adequate hematologic and cardiac status.

Treatment consisted of five different types of regimens, according to the type of cancer:

- Biweekly 5-FU infusion plus folinic acid (FA), LV5FU2, or de Gramont regimen (14), for colorectal carcinomas, in adjuvant or metastatic settings;
- Biweekly 5-FU-FA + irinotecan (FOLFIRI regimen; ref. 15), for metastatic colorectal carcinomas;
- Biweekly 5-FU-FA + oxaliplatin (FOLFOX regimen; ref. 16), for metastatic colorectal carcinomas;
- Four-day continuous infusion 5-FU + cisplatin or carboplatin/3 weeks (17), for head and neck cancers;
- Bolus 5-FU + epirubicin + cyclophosphamide (FEC regimen), every 3 weeks (18), for breast cancers in adjuvant or metastatic settings.

Written informed consent was obtained from all patients as was relevant Ethical Committee approval before starting the trial. Results of pretherapeutic genotyping were not taken into account.

However, the 5-FU dose was individually adjusted in cases of prolonged 5-FU infusion, as it is usually done in some institutions in routine practice (19). It began with the second cycle and was based on the 5-FU plasma concentration measured at the end of the previous 5-FU infusion (i.e., the concentration at the steady state, C_{ss}). 5-FU plasma concentration should be within the optimal therapeutic plasma range, and 5-FU dose was adjusted based on a pharmacokinetic follow up, as previously published (19). Fluorouracil concentrations in plasma were measured in the Oncopharmacology Department. Precision and accuracy tests were carried out beforehand in the control quality test program of the French Clinical Pharmacology Group of the French Federation of Anticancer Centers.

Assessment of Tolerability

Patients were asked for treatment tolerance and underwent examination with hemograms, ionograms, and liver and kidney tests, before every chemotherapy cycle. All early 5-FU-related adverse events (i.e., at the first or the second 5-FU cycle) were taken in account and graded for severity according to the National Cancer Institute Common Toxicity Criteria scale: diarrhea, mucositis, neutropenia, thrombopenia, central neurotoxicity, cardiac toxicity, and hand-foot syndrome. Treatment had to be stopped in case of grade 3 to 4 toxicity until complete recovery, and in case of grade 4, the French Drug Committee had to be notified.

Methods

Detection of SNPs of *DPYD* and Its Promoter. We selected 22 relevant SNPs previously described in the international literature. These SNPs had either been previously found in patients with DPD activity deficiency, or by their nature and its location were clearly able to generate a deleterious effect on DPD enzyme structure and activity. The SNPs are displayed in Table 1 according to the international nomenclature (20–31). We systematically looked for nine SNPs that had been more frequently reported, in the entire population of 487 patients, and for an additional 13 SNPs, potentially implied in DPD deficiency but more infrequently reported, in 171 patients, with or without early severe 5-FU-induced toxicity.

Construction of Mutant Vectors for Different Coding Regions of *DPYD* Gene. Segments of the DNA of human *DPYD* containing the regions of potential SNPs were amplified by PCR and inserted into pGEMt vector (pGEM-T vector System II, Promega, Madison, WI). Then, competent bacteria were transformed. Mutations were introduced using the QuickChange XL Site-Directed Mutagenesis kit (Stratagene, Amsterdam, The Netherlands). These mutant constructs were sequenced and used to provide homozygous positive controls for further pyrosequencing analysis.

WBC Isolation and DNA Extraction. Blood samples were obtained for DNA isolation and determination of genotypes. All procedures were reviewed and approved by accredited ethics review boards, and patients signed an informed consent form.

Peripheral blood mononuclear cells were isolated by centrifugation of the blood at 3,500 × *g* for 15 minutes. DNA was extracted using DNA Isolation kit for blood/bone marrow/tissue (Roche Molecular Diagnostics, Meylan, France). Each sample was controlled with respect to DNA isolation by UV transillumination of ethidium bromide–stained gels from subsequent electrophoretic separation in 1.2% agarose.

Pyrosequencing Analysis

PCR Conditions. PCRs were done with an initial denaturation for 5 minutes at 95°C followed by 50 cycles of denaturation for 30 seconds at 95°C, primer annealing for 30 seconds at 55°C, and extension for 1 minute at 72°C. All amplification reactions were done in a DNA Thermal Cycler 480 (Perkin-Elmer, Boston, MA) with 1 unit of Taq Polymerase (Euroblue Taq, Eurobio, Les Ulis, France).

PCR conditions were the same for the all mutations tested and produced in each case PCR products without significant background. Table 2 shows the different sets of primers used to amplify the sequences of interest.

Choice of the Sequencing Primers. Different sequencing primers were designed to carry out *DPYD* gene pyrosequencing analysis. Then, a selection was made on the ability to provide interpretable Pyrograms.

Sequencing primers and dispensation order of the nucleotides are displayed in Table 3.

Conditions for the Pyrosequencing Analysis. Templates for the pyrosequencing analysis were prepared as recommended by the manufacturer and as previously described (13, 32, 33).

5-FU Plasma Clearance

5-FU plasma clearance was calculated at the end of 5-FU infusion (i.e., at the steady state) because 5-FU half-life is about 8 to 10 minutes, according to the

Table 1. Characteristics, locations, and effect on *DPYD* enzyme activity of the different SNPs previously described in the literature

Name (ref.)	Exon	Nucleotide	Amino acid	Frequency (%)	Consequence
SNPs systematically searched for					
DPYD (21) promoter		−1590T>C		0.5	IFNγ-binding site altered, lowered expression
DPYD 9A (22–24)	2	85T>C	C29R	29	Consequence unclear
DPYD 7 (25)	4	295-298 del TCAT	Frameshift	U	Nonsense, truncated protein, 0 activity
DPYD 7 (26)	11	1156G>T	E386Ter	U	Nonsense, truncated protein, 0 activity
DPYD 7 (24, 27, 28)	13	1679T>G	I560S	U	Missense, FAD site, interference with cofactor binding, no activity
DPYD 2A, G1A (11)	14	IVS14 + 1G>A	Exon 14 skipping,	1.5	Splice site mutation, uracil-binding site deleted
DPYD 9B (22)	21	2657G>A	R886H	U	Missense, conformational site modified
DPYD 9B (10, 27, 29)	22	2846A>T	D949V	U	Missense, direct interference with cofactor binding or electron transport, [4Fe-4S] function altered
DPYD 10 (10)	23	2983G>T	V995F	U	Missense
SNPs searched for in case of grade 3 to 4 toxicity and upper SNPs negative					
DPYD 10 (28)	2	61C>T	R21X	U	Nonsense, truncated protein, no activity
DPYD 10 (28)	2	62G>A	R21Q	U	Missense, no consequence?
DPYD 10 (30)	4	257C>T	P86L	U	Missense mutation, direct interference with electron transport, [4Fe-4S] nonfunctional
DPYD 10 (30)	6	601A>C	S201R	U	Missense, direct interference with electron transport
DPYD 10 (30)	6	632A>G	Y211C	U	Enzyme structure altered, enzyme destabilization
DPYD8 (27)	7	703C>T	R235W	U	Missense, FAD site affected
DPYD8 (31)	8	812 del T	Frameshift		Truncated protein, no activity
DPYD8 (10)	10	1039-1042 del TG	Frameshift	U	Nonsense, NADPH site affected, no activity
DPYD8 (27)	10	1003G>T	V 335L	U	Missense, NADP site affected
DPYD8 (30)	12	1475C>T	S492L	U	Missense, FAD site, interference with cofactor-binding, no activity
DPYD 4 (24)	13	1601G>A	S534N	0.8	Missense, between FAD and uracil-binding site, reduced activity
DPYD 3 (30)	14	1897delC	Frameshift	U	Stopping codon before uracil-binding site
DPYD 3 (10)	23	2933A>G	H978R	U	Missense, direct interference with cofactor- binding or electron transport, [4Fe-4S]

Abbreviation: U, unreported in the literature.

following formula: Cl = flow rate (mg m^{-2} h^{-1}) / C_{ss} (mg L^{-1}), where C_{ss} is the 5-FU concentration at the steady state.

5-FU concentrations in plasma were determined with a previously described method (19, 34).

Statistical Analysis

Frequencies of SNPs and toxic side effects were measured and then compared. Correlations were looked for to assess whether or not statistical difference existed between two observations (i.e., between two percentages relative to toxic events occurrence). Pearson's χ^2 test was used.

We determined the sensitivity, specificity, and positive and negative predictive values of SNPs as prediction factors of toxic side effects. Sensitivity (Se) was calculated as follows: number of true positive patients/number of true positive + false-negative patients.

Specificity (Sp) was calculated as follows: number of true negative patients/number of true negative + false-positive patients; positive predictive value was calculated as follows: Se × Pre/Se × Pre + (1 − Sp) (1 − Pre); negative predictive values was calculated as follows: Sp(1 − Pre) / Sp(1 − Pre) + Pre(1 − Se), where Se = sensitivity, Sp = specificity, and Pre = prevalence.

Analyses were done using SPSS software (SPSS, Paris, France).

Table 2. Primers sequences of eight systematically searched *DPYD* gene SNPs in the study

DPYD SNPs	Sequencing primers	T_m
IVS1 + 1G A	AGGCTGACTTTCCAGA	48
−1590T>C	ATTTGCAAAGGTAAGATACT	49.1
2846A>T	GCAAGTTGTGGCTATGA	50.4
85T>C	CAAACTCATGCAACTCTG	51.4
1156G>T	TGCTAAGGAAGAAAAGTGT	50.2
2657G>A	TTGGACCTTATCTGGAA	47.9
2983G>T	GCTGTACTCTGTGTCTCAGT	57.3
295-298 del TCAT	CCAACTAATCTTGATATTAA	47.1

Results

Technical Results

Conditions for the Pyrosequencing Analysis. To sequence the 22 variants, the sequencing primers designed were suitable for our method, and no interfering peaks were generated. The detection of the 22 variants used standards variables and provided satisfactory results both for the wild type detected in patients as for the mutant sequence from the constructs, engineered to harbor a variant sequence, as illustrated by pyrograms of IVS14 + G>A, 2846A>T, and 1679T>G (Fig. 1).

Clinical Results

Four hundred eighty-seven patients were studied (166 women and 321 men), whose mean age was 63 ± 11.5 years (range = 23–88 years), treated for either colorectal, head and neck, gastric, or breast cancer, in an adjuvant setting or with first-line therapy for metastatic disease. All patients received 5-FU-based chemotherapy according to five different schedules: 168 patients received 5-FU alone (de Gramont regimen; ref. 14); 99 patients were treated with 5-FU plus irinotecan (FOLFIRI; ref. 15); 91 patients received 5-FU plus oxaliplatin (FOLFOX 4; ref. 16); 99 patients were treated with 5-FU plus cisplatin, in a 3 weekly regimen (17); and 30 patients had the FEC regimen (18).

5-FU-related toxicities are displayed in Table 4. Of the 487 patients, 20 had grade 4 toxic side effects, in either the first or the second cycle, consisting principally of diarrhea and neutropenia. In these cases, toxic effects comprised a polyvisceral syndrome. Two of the 20 patients experienced grade 4 toxicity despite an initial 5-FU dose reduction of 20% and 40%. Fourteen of the 20 patients who had grade 4 toxicity had to be transferred to an intensive care medicine department for 15 to 28 days because of a polyvisceral toxic syndrome, consisting of diarrhea, febrile neutropenia and mucositis. Four of these 14 patients had neurologic symptoms with coma. Treatment had to be stopped for 8 of the 20 patients for grade 4 toxicity but was continued in 11 patients, following a variable 5-FU dose reduction from 15% to 75% of the initial dosage, followed by an individual 5-FU dose adjustment with pharmacokinetic follow-up.

Twenty-four patients experienced early grade 3 toxic side effects in either the first or second cycle, again principally diarrhea and neutropenia. Of these 20 four patients, treatment had to be stopped in two patients but could be continued in the other 22, with a 15% to 50% 5-FU dose reduction and pharmacokinetic follow-up.

Fourteen and nine patients had grade 2 or 1 toxic side effects, respectively, as early as the first cycle, and these had an individual dose adjustment, with 15% to 45% and 20% to 35% dose reductions, respectively.

We assessed whether or not the distribution of early toxicity depended on the chemotherapy regimen and concluded that toxicity in the first and second cycles was independent of the regimen used. The frequencies of grade 3 to 4 toxic side effects were equivalent with the 5-FU + FA, FOLFIRI, and FOLFOX regimens (8.3%, 6%, and

Table 3. Characteristics of the eight main primers used for PCR

SNPs	Primers sequences	Sense	T_m	Amplification length
IVS1 + 1G A	ATCAGTGAGAAAACGGCTGC	Sense	60	150
	TAAACATTCACCAACTTATGCCA	Antisense	52	
−1590T>C	ATCCAGGACTCTCCCCAGAT	Sense	59.4	201
	CCTTGGTCAGATCCCAGAAG	Antisense	59.4	
2846A>T	AAGCACTGCAGTACCTTGGAA	Sense	57.9	101
	TCATGTAGCATTTACCACAGTTGA	Antisense	57.6	
85T>C	CCTGGCTTTAAATCCTCGAA	Sense	55.3	98
	TCTTATCAGGATTTCTTTTCCAATG	Antisense	56.4	
1156G>T	TGGAACTTGCTAAGGAAGAAAA	Sense	54.7	124
	AACCAAAGGCACTGATGACC	Antisense	57.3	
2657G>A	TCTGACCTAACATGCTTC	Sense	51.4	100
	AGGCCTTTTGGGGATAAAAC	Antisense	55.3	
2983G>T	TTTGATCCAGAAACCCACCT	Sense	55.3	139
	AGGGTACGCCTCTCTTTGGT	Antisense	59.4	
Del TCAT	TGTCAGAAGAGCTGTCCAA	Sense	54.5	62
	TTGTTTGCAATACTTGTGATG	Antisense	52	

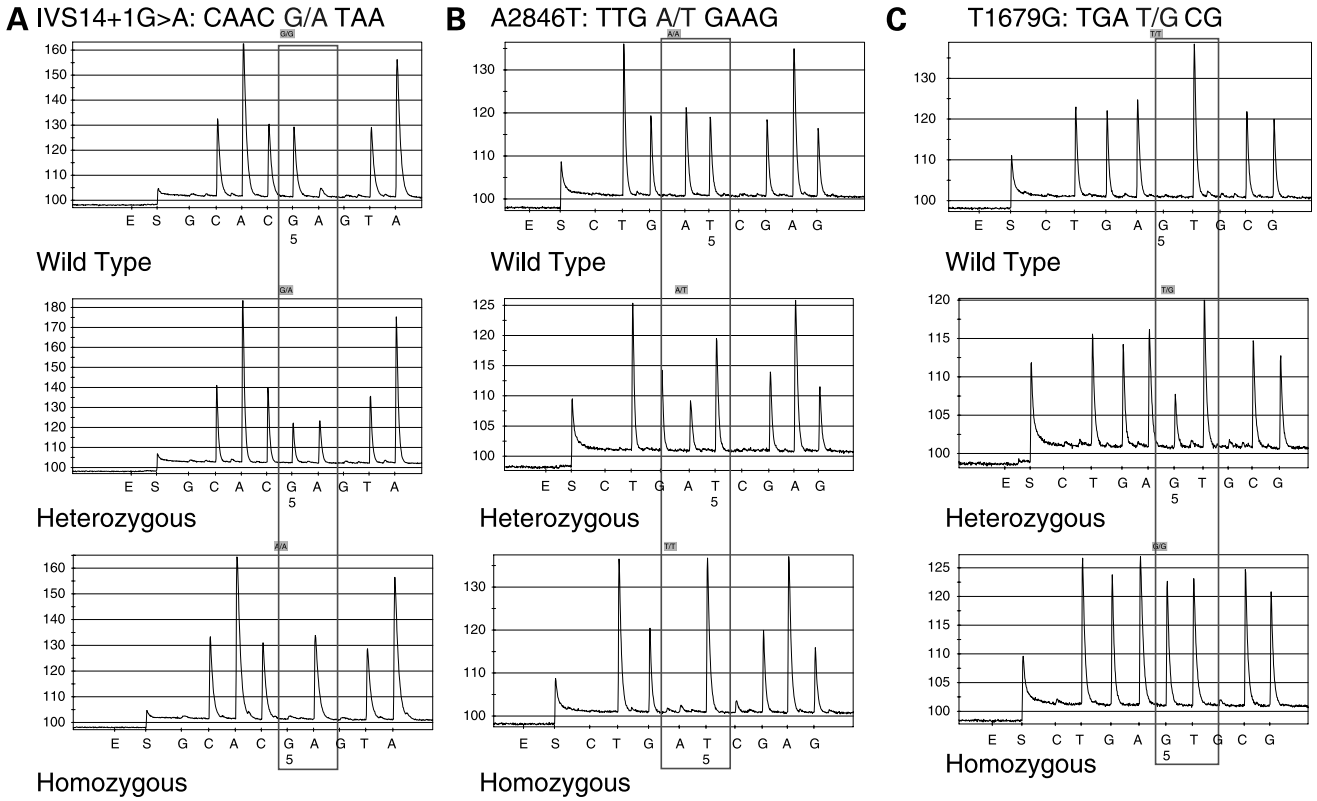

Figure 1. Pyrograms of IVS14 + G>A (**A**), A2846T (**B**), and T1679G (**C**) SNPs from patients.

5.5%, respectively). Although the toxicities were somewhat little higher in patients receiving the 5-FU-CDDP regimen (16%), it was felt that this was a result of the combination, and the resultant increased incidence of hematotoxicity.

Concerning *DPYD* gene polymorphism, nine SNPs were systematically searched for in the whole population of patients, whatever the tolerance of the treatment (Table 5). Ten patients had IVS14 + 1G>A polymorphism. In nine of these, sequencing showed a heterozygous result. These nine included three women and five men, as well as one man whose DNA was shown to be heterozygous both for the IVS14 + 1G>A and the −1590T>C SNPs. In one woman, sequencing showed a homozygous result. In 10 patients (4 women and 6 men), a heterozygous result was observed for the 2846A>T SNP. In 150 and 15 patients, heterozygous and homozygous results, respectively, were found for the 85T>C SNP. DNA in seven patients was heterozygous for the −1590T>C SNP. One patient's DNA was heterozygous for 1679T>G.

Table 4. Grade distribution and characteristics of the early toxic side effects (cycles 1 and 2) in the population of patients

	Toxicity grade				
	4	3	2	1	0
Diarrhea	11	9	6	6	
Neutropenia	11	8	—	1	
H-F syndrome	2	3	4	1	
Mucositis	4	2	7	1	
Cardiac toxicity	1	3	—	—	
Nausea, vomiting	2	1	2	—	
All (no. patients), %	20 (4)	24 (5)	14 (3)	9 (2)	421 (86)
Initial dose reduction (%)	40, 19	33, 33, 25	20, 48		50, 40, $(35)_3$, $(30)_2$, $(25)_2$, $(20)_6$, 15

NOTE: At the bottom of the table, the percentage of 5-FU dose reduction at the first cycle for medical reasons. $(\%)_{no.}$ = no. patients when >1.

Table 5. Frequency of different SNPs in our population of 487 patients, frequency of IVS14 + 1G>A, A2846T, and T1679G in our extended population of 1,200 patients, and correlation with toxicity, any grade, and grade 3 to 4

SNPs	Patients heterozygous/ homozygous (of 487 patients)	*n* (%) patients with 1 to 4 NCI grade toxicity	*n* (%) patients with 3 to 4 grade toxicity	Patients heterozygous/ homozygous (of 1,200 patients), frequency	*n* (%) patients with 3 to 4 grade toxicity
IVS14 + 1G>A	9/1	7 (70)	6 (60)	16/1 (1.3/0.1)	11 (70)
2846A>T	10/0	7 (70)	6 (60)	19/0 (1.6)	13 (69)
85T>C	150/15	20 (12)	11 (6.6)	ND	8 (5.5)
−1590T>C	7/0	1 (14)	0	ND	0
1679T>G	1/0	1 (100)	1 (100)	2/0 (0.16)	2 (100)
2983G>T	0/0	X	X	ND	NA
295-298 del TCAT	0/0	X	X	ND	NA
1156G>T	0/0	X	X	ND	NA
2657G>A	0/0	X	X	ND	NA
0 SNP found	300	33 (11)	20 (6.6)	ND	NA

Abbreviations: NCI, National Cancer Institute; ND, not determined; NA, not applicable.

None of the other 13 SNPs that were evaluated were detected in any patient including those with toxic side effects in the first or second cycle and none of the other nine SNPs.

We looked for a correlation between the *DPYD* SNPs and the 5-FU-related toxic side effects (Table 3). Of 10 patients with the IVS14 + 1G>A SNP, six experienced grade 3 to 4 toxic side effect (60%; one FEC, one LV5FU2, two FOLFIRI, one FOLFOX, and one 5-FU-CDDP), presenting principally as a combined polyvisceral syndrome, which was lethal in one patient. One patient, a woman with breast cancer whose sequence analysis was homozygous for IVS14 + 1G>A, experienced severe toxicity after receiving low-dose 5-FU as party of the FEC protocol given in an adjuvant setting. Within a few days of the first cycle, she was hospitalized in intensive care, with grade 4 diarrhea, neutropenia, and mucositis, remaining there for 15 days. Two additional patients with the IVS14 + 1G>A SNP had no toxicity but had an their initial 5-FU dose reduced. Among the six patients with severe toxicity in the first or second cycle, treatment was stopped as early as the second cycle in three and was continued for the other three with a good tolerance following a 25% to 50% dose reduction with pharmacokinetic follow-up.

Similarly, six patients of the 10 with a 2846A>T SNP experienced grade 3 to 4 toxic side effects in the first two cycles (60%; four LV5FU2, one FOLFOX, and one 5-FU-CDDP). Among the three patients without toxicity in the first two cycles, two had a 20% and 45% 5-FU dose reduction. Among the seven patients with initial toxicity, three had their treatment stopped, whereas four received additional 5-FU with 25% to 50% dose reductions and pharmacokinetic follow up.

One patient with a 1679T>G SNP experienced grade 4 toxicity (LV5FU2). After a 6-week treatment interruption, 5-FU was safely reintroduced with individual pharmacokinetic adjustment.

One hundred sixty-five patients presented with the 85T>C SNP (150 heterozygous and 15 homozygous). Of these, 11 had grade 3 to 4 toxicity (6.7%); none of them was homozygous. Seven patients had the −1590T>C SNP. Only one of these seven experienced grade 1 toxicity in the first cycle, and this was a patient in whom a IVS14 + 1G>A SNPs was also noted.

For 300 patients, a SNP was not detected (62%). Thirty-three experienced early toxic side effects of any grade in the first or second cycle (11%), whereas 20 had severe grade 3 to 4 toxicities (6.7%). Of the seven patients who had grade 4 toxic side effects, treatment was stopped for three and continued for four, with dose reductions from −20% to −40%. The therapy tolerance of those whose dose was reduced was excellent. Thirteen patients experienced grade 3 toxicity. Among them, treatment was stopped for two patients and continued for the others with −15% to −40% dose reductions and individual pharmacokinetic follow-up.

We compared the frequency of grade 3 to 4 toxic side effects according to the presence of a SNP and the type of SNP. We found a highly significant difference in toxicity when patients with either a IVS14 + 1G>A, 2846A>T, or 1679T>G SNP were compared with those without a SNP ($P = 10^{-6}$). We pooled these three SNPs even if we could not draw any statistical conclusion about the statistical correlation between 1679T>G and severe toxicity because this SNP was shown to modify the FAD site, to interference with cofactor binding, and to suppress DPD activity. We found no difference between −1590T>C or 85T>C patients and patients without a SNP ($P = 0.9$). The presence of one of the three relevant SNPs (IVS14 + 1G>A, 2846A>T, and 1679T>G) was highly correlated with early severe toxicity ($P = 2.8\ 10^{-10}$).

Sensitivity, specificity, and positive and negative predictive values of the detection of these three major SNPs (IVS14 + 1G>A, 2846A>T, or 1679T>G) as predictive factors for toxic side effects were 0.31, 0.98, and 0.62 and 0.94, respectively.

5-FU plasma clearance at the first cycle was evaluated in 457 patients. The clearance was not evaluated in the

FEC regimen, where 5-FU was given in bolus infusion. We compared the clearances of the patients with IVS14 + 1G>A, 2846A>T, or 1679T>G SNP with those of the patients with no SNP found or with 85T>C or −1590T>C (Table 6). Mean clearances were 74.9 ± 38.3 L h^{-1} m^{-2} (range = 21.2–183.5) and 132.3 ± 46.6 L h^{-1} m^{-2} (range = 36.8–369.7), respectively. Median values were 66.2 and 123.9 L h^{-1} m^{-2}, respectively. They were statistically different ($P < 0.001$). Because some patients (seven with measurable clearances) with IVS14 + 1G>A, 2846A>T, or 1679T>G SNP had no or light or mild toxic side effects (0 to II), and because some patients with no SNP found or with 85T>C or −1590T>C (26 with measurable clearances) experienced severe toxic side effects, we compared the clearances in these two populations of patients. Mean clearances were 95.5 ± 47.5 L h^{-1} m^{-2} (range = 21.2–183.5) and 77.5 ± 21.1 L h^{-1} m^{-2} (range = 36.8–119.5), respectively. Median values were 90.7 and 76.6 L h^{-1} m^{-2}, respectively. Interestingly, we found no statistical difference ($P = 0.156$).

Discussion

In the present prospective study, we determined frequencies and effects of certain *DPYD* SNPs on 5-FU tolerance and evaluated a high-throughput genotyping method for detection of patients at risk of severe 5-FU-induced toxic side effects. Twenty percent to 30% of grade 3 to 4 toxic side effects and 0.5% mortality rate have been reported in patients treated for advanced diseases or in adjuvant therapy with 5-FU bolus administration or continuous infusion (14–16, 34, 35). They have been investigated for their relationship with 5-FU metabolism rate and/or DPD activity deficiency, but no prevention strategy has been widely proposed and accepted (2, 3, 35). Certain *DPYD* SNPs can have a deleterious effect on DPD activity, if they are located in some hotspots of the gene, for example, interfering with substrate or cofactor binding, with electron transport by altering [4Fe-4S] function, or resulting in truncated nonfunctional protein (10, 24, 30, 36, 37). According to the literature, the prevalence of *DPYD* SNP heterozygous patients at risk of severe fluoropyrimidine's toxicity is about 3% to 5%, the splice site mutation IVS14 + 1G>A being by far considered the most common one, with 52% frequency compared with the other ones (12). However, until now, the exact determination of relevant *DPYD* alleles, their respective frequencies, and their effects on DPD activity and patients' tolerance to 5-FU, remained to be determined (34).

Different pharmacologic approaches have been tested for detecting DPD deficiency, such as the DPD activity measurement in peripheral mononuclear cells by radioenzymatic assay (38, 39), the measurement of natural pyrimidines concentrations in plasma (40), the determination of the ratio dihydrouracil/uracil (UH_2/U) in plasma (41), and the 2-^{13}C-uracil breath test (42). The detection of relevant *DPYD* gene SNPs by genotyping technique is promising, but many questions remain unresolved, such as which adequate method and which relevant SNPs (43).

In the present prospective study, we set up a high-throughput minisequencing method by pyrosequencing technique for genotyping *DPYD* gene on a large population of patients, suitable to a clinical practice. We were able to detect 22 SNPs. First, in our whole population of 487 Caucasian patients, we looked for nine SNPs, eight of the gene itself in the coding regions and one in its promoter, because of both their frequency and their potential deleterious effect on DPD activity. Then, we systematically searched for the 13 other ones: first, in 44 patients with early 5-FU-related toxicity but no SNP found and, because we found none of them, we continued for a total of 171 patients, and at last we stopped because the minimum frequency of a SNP is usually estimated at 1%.

Table 6. 5-FU plasma clearance function of the presence or absence or *DPYD* gene variants and clinical toxicity

SNPs	5-FU clearance for patient with toxicity NCI grade (0-1-2)				5-FU clearance for patient with toxicity NCI grade 3 to 4			
		n	Mean	SD		*n*	Mean	SD
IVS14 + 1G>A	h	4	72.11	44.62	h	4	54.32	14.87
					H	1		
2846A>T	h	4	89.68	4.82	h	6	60.76	24.65
1679T>G	h	0			h	1	41.06	
85T>C	h	130	137.13	51.02				
	H	14	137.61	52.39	H	0		
−1590T>C		7	128.49	36.15		0		
2983G>T		0				0		
295-298 del TCAT		0				0		
1156G>T		0				0		
2657G>A		0				0		
0 SNP found		264	136.33	42.26		19	78.67	19.23

Abbreviation: NCI, National Cancer Institute.

In our population of patients, we found five SNPs. IVS14 + 1G>A was the most frequently reported in the literature (11, 12). Its frequency in the present study, both heterozygous and homozygous status, was 1.8% and 0.2%, respectively, quite similar to that previously published (11, 12). Concerning 2846A>T, previous data about its incidence and relevance were very scarce, but we found a high frequency of heterozygosity (2%). Besides, 85T>C frequency was high: 31% heterozygous and 3% homozygous. One patient presented with 1679T>G (0.2%). At last, concerning *DPYD* promoter, seven patients were heterozygous for −1590T>C SNP (1.4%). We found no other SNP.

Of the five *DPYD* SNPs, two were highly correlated with early 5-FU toxicity because 60% of the patients with IVS14 + 1G>A and 2846A>T experienced very early severe toxic side effects. Compared with the risk of toxicity for patients with no SNP found (i.e., 6.6%), the difference was highly significant. For IVS14 + 1G>A, our results are in agreement with previous ones (11, 12). On the contrary, 2846A>T SNP, much less reported in the literature than IVS14 + 1G>A, had a high prevalence in our population of French, White Caucasian patients and was as deleterious as IVS14 + 1G>A on 5-FU tolerance. One patient heterozygous for 1679T>G had grade 4 febrile neutropenia, and his treatment quickly stopped. The frequency in our population of patients is then 0.2%. We are unable to draw any statistical conclusion, but this SNP is known to suppress DPD activity.

We evaluated the effect of these *DPYD* gene variants (IVS14 + 1G>A, 2846A>T, and 1679T>G) on 5-FU pharmacokinetics. Clearly, patients with these SNPs had a significantly reduced 5-FU plasma clearance and were brutally exposed to too high 5-FU plasma concentrations, when its dose was adjusted to body surface area. A close relationship between high 5-FU plasma concentrations and clinical toxicity has previously been shown (34). Thirty percent of patients with either the IVS14 + 1G>A or the 2846A>T SNP had no adverse events. Interestingly, these patients had 5-FU plasma clearance significantly lower than patients with no SNP found and no toxicity but a little higher than patients with SNPs and toxicity. In these cases, we can hypothesize that toxicity did not occur because of either an initial 5-FU dose reduction and/or a pharmacokinetic follow-up. On the other hand, patients with no SNP found, but who experienced severe initial toxic side effects, had a significantly reduced 5-FU plasma clearance.

We would point out the following. Even after severe toxicity, treatment could be safely resumed, in 50% of the patients, provided the dose was reduced, and close pharmacokinetic monitoring was instituted to avoid too high 5-FU plasma levels. Thus, the identification of genetic heterozygosity at either IVS14 + 1G>A or 2846A>T is not an absolute contraindication to 5-FU administration, provided the physician is aware and appropriate precautions are instituted.

We would also note that although the 85T>C SNP was found at a high frequency in our population (31% heterozygous and 3% homozygous), this SNP seems to have had little to no effect on 5-FU tolerance because only 11 of 165 (6.6%) patients had severe toxic side effects. Although previous studies have reached contradictory conclusions about its possible role in 5-FU toxicity (23, 32), our results agree with those of Collie-Duguid et al., in that we would characterize it as a common and pharmacokinetically inconsequential polymorphism (32). Similarly, although the −1590T>C SNP, located on the IFN-binding site of the *DPYD* promoter, has been previously suspected of lowering *DPYD* gene expression, our results suggest it has little to no clinical effect. In our population, only one of the seven patients experienced toxicity, and this patient was found to have SNPs at both T-1590C and IVS14 + 1G>A.

Finally, we would point out that in 300 patients representing 62% of the total population, a mutation could not be found at any of the 22 SNPs screened. Twenty of these 300 patients or 6.6% had severe toxicity in either the first or the second cycle. In three of these 20 patients, treatment was stopped, whereas in the other 17 patients, treatment was resumed after they had recovered from the toxic side effects with secondary dose reductions of 15% to 40%, according to a pharmacokinetic follow-up protocol. Although the incidence of toxicity in these patients was substantially less than in patients with some of the SNPs, these result raises the question of the cause of 5-FU toxicity in these patients. Possible explanations include (*a*) additional *DPYD* mutations (SNPs) that can affect pharmacokinetics may yet remain to be discovered, (*b*) Mutations or SNPs in other enzymes, involved in 5-FU catabolism, may be causative. For one such enzyme, dyhydropyrimidinase, a deficiency has been rarely reported (44). (*c*) Polymorphism in thymidylate synthetase, a target of 5-FU, may be etiologic. A polymorphism in the thymidylate synthetase gene promoter (2R/2R and 3R/3R) has been correlated with toxicity, although usually delayed and chronic in its presentation (45). Finally, (*d*) epigenetic factors not yet identified may be responsible.

We would argue that given the large number of patients treated each year with 5-FU or other fluoropyrimidines, such as capecitabine and UFT, and the human and economical cost of grade 3 and 4 toxic side effects, pretherapeutic detection of DPD deficiency should be considered. *DPYD* genotyping to detect the IVS14 + 1G>A, 2846A>T, and 1679T>G SNPs is a promising approach. We would argue that detection of only the IVS14 + 1G>A SNP is insufficient. As for the other SNPs that seem to be much rarer but clinically relevant, we would suggest that evaluation may depend on the country one is dealing with because their prevalence may vary among different populations. In practice, one may ask whether all known relevant SNPs, or merely those that are found more frequent, should be evaluated. We would note that because pyrosequencing can give results given within a day, a more comprehensive screen may be suitable for clinical practice. However, we would caution that *DPYD* SNP detection cannot prevent all severe toxic side effects that occur in the first or second cycle. In our experience, *DPYD* SNP detection would seem to be able to avoid at

least 40% of 5-FU early severe toxicities. Although the sensitivity of this test at 0.31 is low, its positive predictive value at 0.62 is good, and its specificity (0.98) and its negative predictive value (0.94) are very high.

The pretherapeutic detection of DPD deficiency could reap benefits in terms of reduced costs and more importantly reduced toxicity with improved quality of life. We feel that this test will have to be compared, in terms of suitability to clinical practice, sensitivity, specificity, and positive and negative predictive values, as well as cost, with other approaches, such as the pretherapy uracil plasma measurement, UH_2/U plasma ratio determination, or more recently reported, the 2-^{13}C-uracil breath test, although 2-^{13}C-uracil is not currently available in every country (42).

We would also point out that one should not simply rely a detection of a SNP or a DPD deficiency. The management of a patient with DPD deficiency depends on his homozygous or heterozygous variant status and on other personal variables, such as age, comorbidity factors, and disease staging. It requires a therapeutic and pharmacologic advice and a close collaboration among physicians, pharmacologists, and molecular biologists. In case of homozygosity, an alternative treatment is recommended, 5-FU metabolism being quite totally impaired. In case of heterozygosity, 5-FU can be safely and efficiently given for several months, with added strict safety precautions, such as an initial 5-FU dosage reduction and an individual dose adjustment based on a close clinical and pharmacokinetic follow-up (19, 20).

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