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5-Fluorouracil-related severe toxicity: A comparison of different methods for the pretherapeutic detection of dihydropyrimidine dehydrogenase deficiency

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Abstract

5-Fluorouracil (5-FU)-related early toxicity, due to a metabolic deficiency, is rare but is potentially severe and even lethal (0.1%). It is due to dihydropyrimidine dehydrogenase (DPYD) gene polymorphism or some epigenetic factors. The detection of metabolic change could prevent severe toxicity, but until now it has not been carried out in clinical practice.

Purpose: To find the simplest and most accurate pretherapeutic test to predict DPD deficiency in patients treated with 5-FU by comparing different approaches.

Results: Two hundred and fifty two French Caucasian patients treated by 5-FU infusion were studied. A two-step strategy, combining firstly SNP detection and uracil plasma measurement, followed, in cases where metabolic deficiency was suspected, by dihydrouracil/uracil ratio determination to confirm deficiency and to determine the optimum 5-FU dosage, appeared the best approach, with 83% and 82% sensitivity and specificity, respectively.

Conclusion: These data support the future use of this approach, suitable to clinical practice, as screening test to identify DPD deficiency before 5-FU-based therapy.

Keywords: 5-Fluorouracil; Fluoropyrimidines; Dihydropyrimidine dehydrogenase; Deficiency; Toxicity; Single nucleotide polymorphism

1. Introduction

5-Fluorouracil can result in severe toxic side-effects, including death, in advanced or adjuvant setting, due to catabolic pathway deficiency [1–3]. Dihydropyrimidine dehydrogenase (DPD), the rate-controlling enzyme of endogenous pyrimidine and fluoropyrimidine catabolism, is subject to a genetic polymorphism and its activity shows a wide range of individual variation [4–6]. This results in a broad range of enzymatic deficiency from partial (3–5% of the population) to complete loss (0.2%

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of the population) of enzyme activity and consequently to severe polyvisceral 5-FU-induced toxicity [7,8].

Considering the common use of 5-FU, and oral fluoropyrimidines, pretreatment detection of DPD deficiency could prevent severe toxicity.

To date, different techniques have been reported. They can be arranged according to their check point, from the gene encoding the DPD enzyme to the 5-FU catabolic pathway: (1) the detection of relevant DPYD gene SNPs; (2) the level of DPYD mRNA expression; (3) the evaluation of DPD activity in lymphocytes with radio-enzymatic techniques; (4) the measurement of uracil, a natural substrate of DPD, in plasma or urine; (5) the UH_2/U ratio in plasma catabolite (dihydrouracil) and substrate (uracil) of DPD; (6) the more recently the [2-C^{13}]uracil breath test; (7) the evaluation of 5-FU plasma clearance during treatment for a pharmacokinetic follow-up and an individual 5-FU dosage adjustment.

These last approaches allow for the use of individual dose adjustment but cannot detect major DPD deficiency prior to treatment, and thus cannot prevent immediate severe toxicity [9]. It is suitable for the avoidance of toxicity in patients with a known DPD deficiency, and for dose intensification in underdosed patients [10].

The determination of uracil in plasma or urine has been reported previously [11]. However, its levels can be influenced by factors other than DPD activity, such as nutrition and pyrimidine production; no threshold has been reported in the literature to date, and the correlation between uracil levels and 5-FU plasma clearance remains to be measured. To get rid of potential interfering factors, we simultaneously measured both plasma uracil and its dihydrogenated metabolite and characterized the dihydrouracil/uracil ratio (UH_2/U) prior to treatment. We found a good correlation with 5-FU plasma clearance and this ratio was later correlated with DPD activity [12,13].

DPD activity measured in peripheral mononuclear cells by radio-enzymatic assay has been considered by some authors as the reference technique to detect severe DPD deficiency [14,15]. Nevertheless, it is clearly a tedious, time-consuming technique, requiring large volumes of blood and radiolabelled materials, and is hence not suitable for clinical practice.

Genotyping techniques can be used for DPYD gene polymorphism detection [16]. Thus far, at least thirty variant DPYD alleles have been published, with or without a deleterious impact upon DPD activity [17]. Splice-site mutation IVS14 + 1G > A is considered as the most common mutation (52%) [18]. For most of the other ones, their location and hypothetical interference with the functional status of enzymes has been described, but frequently no correlation to toxicity was looked for and their respective frequencies often remain unknown [19]. A few genotyping methods such as RFLP, and DHPLC have been reported, though they are often not suitable for the rapid detection of different DPYD gene mutations within an acceptable timeframe for routine practice.

The uracil breath test has been recently correlated with DPD deficiency but [2-C^{13}]uracil is not currently available in many countries [20].

Therefore, the objective of the present study was to determine the most accurate and simple method to predict DPD deficiency before 5-FU-based treatment.

We compared 4 different methods in a population of French Caucasian patients:

1. Uracil plasma measurement, by liquid chromatography [21],
2. Dihydrouracil/uracil ratios in plasma, by liquid chromatography [22],
3. Quantification of DPD mRNA in lymphocytes, in real-time quantitative RT-PCR,
4. Detection of DPD genes SNPs in lymphocytes, using a minisequencing method via pyrosequencing techniques [23].

These parameters were correlated to individual 5-FU metabolism, the 5-FU plasma clearance at the first cycle, and to tolerance to the treatment.

We selected neither the radio-enzymatic method since it obviously cannot be used on a large scale nor the breath test because [2-C^{13}]uracil is not available everywhere.

2. Patients and treatments

This prospective study was conducted on 252 French Caucasian patients treated for advanced colorectal carcinomas or in the adjuvant setting by 5-FU – leucovorin chemotherapy regimen. To be eligible, patients had to be naïve of 5-FU, have a WHO performance status (PS) < 2, a life-expectancy of at least 3 months, an age lower than 80 years, and adequate hematological and cardiac status.

Treatment consisted of two types of regimens:

1. Biweekly 5-FU 400 mg/m^2 bolus + 2500 mg/m^2 46 h infusion plus 400 mg/m^2 folinic acid (FA), LV5FU2 or de Gramont modified regimen [24],
2. Weekly 5-FU 1200 mg/m^2, 4 h infusion plus 200 mg/m^2 folinic acid [9].

Written informed consent was obtained from all patients as was relevant Ethical Committee approval before starting the study.

Results of pretherapeutic DPD deficiency detection were not taken in account. However, 5-FU doses were individually adjusted as it is usually done in some institutions in routine practice. 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. 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 [9,10]. 5-Fluorouracil concentrations in plasma were measured in the Oncopharmacology Department [21]. 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'.

2.1. Tolerance assessment

Patients were asked for treatment tolerance and underwent examination with hemograms, ionograms, 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, to make sure as much as possible that they were due to a severe 5-FU metabolic pathway deficiency, and graded for severity according to the NCI–CTC scale: diarrhea, mucositis, neutropenia, thrombopenia, central neurotoxicity, cardiac toxicity, and hand-foot syndrome. Treatment had to be stopped in case of grade 4 toxicity and the French Drug Committee had to be notified.

2.2. Methods

Before the first 5-FU infusion, 5 ml blood-samples were collected in heparinized tubes; at the same time blood was withdrawn for the usual exams: hemogram, liver and renal function tests. Plasma and cells were immediately separated by centrifugation (2500*g*) at 4 °C and stored at −70 °C for subsequent analysis.

1. *Uracil measurement in plasma.* Uracil concentrations (μg/L) in plasma were measured according to a liquid chromatography method as previously described [21].

2. *Dihydrouracil/uracil ratio in plasma (UH_2/U).* Both dihydrouracil (UH_2) and uracil (U) concentrations (μg/L) were simultaneously determined in plasma by an improved liquid chromatography method, and their ratios were assessed as previously described [22].

3. *Quantification of DPYD mRNA in lymphocytes.* The quantification of DPYD mRNA was performed according to a real-time quantitative RT-PCR as previously published [25].

4. *Detection of SNPs of DPYD and its promoter.* The detection of SNPs was performed according to a real-time pyrosequencing method previously published [23].

We systematically looked for relevant SNPs, previously described in the international literature and clearly able to generate a deleterious effect on DPD enzyme structure and activity:

1. IVS14 + 1G > A, so-called G1A (DPYD* 2A), considered as the most frequent SNP, a G–A mutation in the invariant GT splice donor site, leads to the skipping of exon 14, immediately upstream of the mutated splice donor site in the process of DPD pre-mRNA splicing [19,26].
2. 2846A > T so-called D949V, located on exon 22, on the [4Fe–4S] site, electron provider, rarely reported in the literature, interferes directly with cofactor-binding or electron transport [27,28].
3. Del TCAT295-298, so-called DPYD* 7, located on exon 4, results in a codon-stop and finally in a truncated protein and a non-functional enzyme [29].
4. 1156 G > T, so-called E386Ter, located on exon 11, results in a codon-stop and finally in a truncated protein and a non-functional enzyme [30].
5. 2657G > A, so-called R886H (DPYD* 9B) is a missense mutation located on exon 21. This results in a DPD function of 25% that of normal [31].
6. G2983, so-called V995F (DPYD* 10), located on exon 23, close to [4Fe–4S] sites, potentially interferes with electron flux [32].
7. −1590T > C, on the DPYD gene promoter, located in the IFNγ-binding site, potentially results in lowered DPYD expression [33].

2.3. Pyrosequencing analysis

2.3.1. PCR conditions

All amplification reactions were performed in a DNA thermal cycler 480 (Perkin–Elmer, Boston, USA) with 1 U of *taq* polymerase (Euroblue Taq – Eurobio, Les Ulis, France). PCR conditions were the same for the 7 tested mutations.

2.3.2. 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™. DNA products consisted of amplified genomic DNA from control subjects.

2.3.3. Conditions for the pyrosequencing analysis (34, 35)

Templates for the pyrosequencing analysis were prepared as recommended by the manufacturer. Real-time pyrosequencing was performed at 28 °C in an automated 96-well pyrosequencer using PSQ SNP 96 enzymes and substrates (Pyrosequencing AB, Uppsala, Sweden). Prior to analysis, the enzymes and each of the four dNTPs (PSQ 96 SNP Reagent Kit, Pyrosequencing AB, Uppsala, Sweden) were loaded into a special cartridge that was mounted in the PSQ instrument.

5. *5-FU plasma clearance.* 5-FU plasma clearance was calculated at the end of 5-FU infusion, i.e. at the steady state, since 5-FU half-life is about 8–10 min, according to the following formula [9]:

$Cl = \text{Flow rate (mg m}^{-2}\text{ h}^{-1}\text{)/Css (mg L}^{-1}\text{)}$

Where Css = 5-FU concentration at the steady state.

5-FU concentrations in plasma were determined by HPLC with a previously described method [21].

2.4. Statistical analysis

Correlations were looked for in order to assess whether or not statistical differences existed between the two observations, i.e. between two percentages relative to toxic event occurrence. Pearson Chi-square test was used. The level of significance was set *a priori* at $p < 0.05$. Analyses were performed using SPSS software (SPSS, Paris, France). In order to determine the distribution pattern of 5-FU clearance and UH_2/U ratio in the patient population, we tested the hypothesis that the sample population distribution followed a normal distribution, by χ^2 analysis, using parametric statistical tests, including the mean, median, SD and the 95% distribution range. Correlations between pharmacokinetic parameters were assessed by simple linear regression analysis. The level of significance was set at $p < 0.05$.

To evaluate each pretherapeutic parameter as a predictive factor of toxic side-effects, we determined sensitivity (Se), specificity (Sp), and positive and negative predictive values of the different tests. First, to check that the tests ordered the patients in true +, true −, wrong + , and wrong −, we crossed the tests and calculated the κ concordance coefficient. This coefficient allows the independence of the two tests to be determined. Then, we determined the threshold values for continuous variables, uracil and UH_2/U for predicting toxicity, using receiver operating characteristic. The receiver operating characteristic (ROC), also receiver operating curve, is a graphical plot of the sensitivity *vs* (1 − specificity) for a binary classifier system as its discrimination threshold is varied. The threshold is choose as above which the test is considered to be abnormal and below which it is considered to be normal. The position of the threshold will determine the number of true +, true −, false +, and false −. Then we determined the threshold as the point for which sensitivity and specificity were maximum.

3. Results

3.1. Number and clinical characteristics and outcomes of the patients

Two hundred and fifty two patients were studied, 112 females and 140 men, their mean age being 67 ± 11.4 years old, (30–80). Their performance status was 220 PS 0-1, and 32 PS 2. Ninety-nine out of 168 in the LV5FU2 group were treated for metastatic cancer, *vs* 27 out of 84 in the FuFol group.

5-FU-induced toxic side-effects were graduated (Table 1). Early toxic side effect frequency was 11.3%, with 6.3% at toxicity grades III–IV. We found no difference between the two kinds of 5-FU administrations, both in term of toxic side-effects frequency and grading (Table 1).

The main toxic side-effects were diarrhoea (52.4%) and hand-foot syndrome (66.7%). Neutropenia mucositis and thrombopenia frequencies were 19.0%, 14.3%, and 4.8%, respectively.

Five patients had to be hospitalized from 8 to 40 days, due to severe multivisceral toxic side-effects, and 2 of them died of toxicity 15 and 30 days after 5-FU infusion, despite being transferred to the intensive care department. Three other patients had their treatment stopped due to grade III–IV toxicity. Ten patients with grade III–IV toxicity continued their treatment with a reduced dose

Table 1
Frequency of toxic side-effects for the first 2 cycles, according to their NCI–CTC grade

			Toxicity									
Toxicity grade	Grade 0		Grade I		Grade II		Grade III		Grade IV		Grades I–IV	
Regimens	LV5FU2	FuFol	LV5FU2	FuFol	LV5FU2	FuFol	$LV5FU_2$	FuFol	LV5FU2	FuFol	$LV5FU_2$	FuFol
Nb of patients	138	71	4	2	5	1	4	2	6	3	19	8
Frequency (%)	88.5	88.7	2.6	2.5	3.2	1.3	2.6	2.5	3.8	3.8	12.2	10.0

from 10 to 50%, calculated from a pharmacokinetic follow-up.

In order to look for correlations between 5-FU toxicity and predictive parameters, patients were pooled in 2 groups: (group 1) no or mild toxicity; grades 0, I and 2 NCI–CTC, and (group 2) severe toxicity: grades III and IV.

3.2. Pharmacokinetic results: determination of 5-FU plasma clearance at the first cycle, pretherapeutic uracil plasma concentrations and UH_2/U ratio distribution

5-FU plasma clearance at the first cycle was evaluated in the 2 groups of patients, in 236 out of 252. It could not be carried out on 16 patients because of infusion flow rate problems or forgotten blood withdrawal. Mean clearances were 100.9 ± 42.9 L h^{-1} m^{-2} [range 21.2–258.1] and 133.4 ± 48.9 L h $^{-1}$ m^{-2} [range 54–365.8] for 5-FU/4 h ($n = 81$) and LV5FU2 ($n = 153$), respectively. Median values were 93.7 L h^{-1} m^{-2} for 5-FU/4 h and 122.7 L h^{-1} m^{-2} for LV5FU2. They were statistically different ($p < 0.001$). Two patients were taken off treatment following the calculation of these mean and median values, because of clearances close to 0 L h^{-1} m^{-2}.

Uracil plasma concentrations before treatment spanned from 5.7 to 70.6 μg/L except for two patients with very high levels: 365 and 3097 μg/L. The mean uracil concentration was 14.9 ± 7.7 μg/L, excluding the two exceptional concentrations, and 28.9 ± 196.3 μg/L when they were included. Median values were 13 μg/L in both cases. Uracil plasma concentration distribution is displayed on Fig. 1. The two patients with very high levels are not shown.

Pretherapeutic UH_2/U plasma ratios were measured in the 252 patients. Their distribution is displayed in Fig. 2. The mean ratio was 7.6 ± 2.7 [0.002–17.3]; the median value was 7.3.

3.3. Genomic results: Determination of DPYD mRNA expression and detection of DPYD gene variants

DPYD mRNA expression was quantified in 118 patients from samples withdrawn prior to 5-FU treatment. Results was weighed by G3PDH expression. The mean expression was 0.22 ± 0.13 [range 0.01–0.70] with a median of 0.20.

Concerning genotyping, 4 different SNPs were found in 93 patients out of our population of 252 patients: 2846A > T (8 heterozygote patients, 3.2%), IVS14 + 1G > A (3 heterozygote patients, 1.2%), 1590T > C (3 heterozygote patients, 1.2%), and 85 T > C SNP (69 heterozygote patients, 27.4% and 10 homozygote patients, 4%).

Four patients had 2 or 3 simultaneous different SNPs on the DPYD gene:

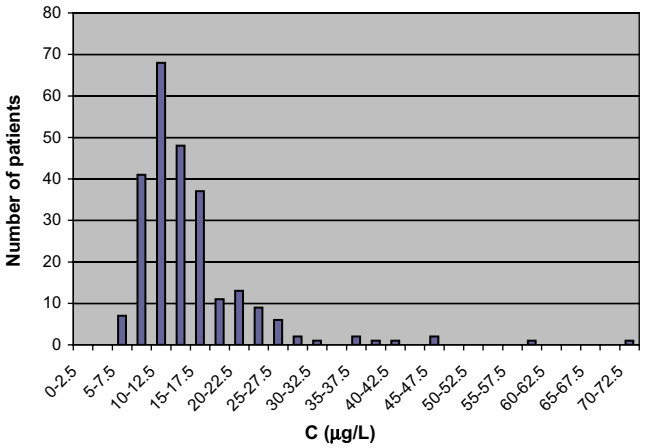

Fig. 1. Pretherapeutic distribution of uracil plasma concentrations (μg/L).

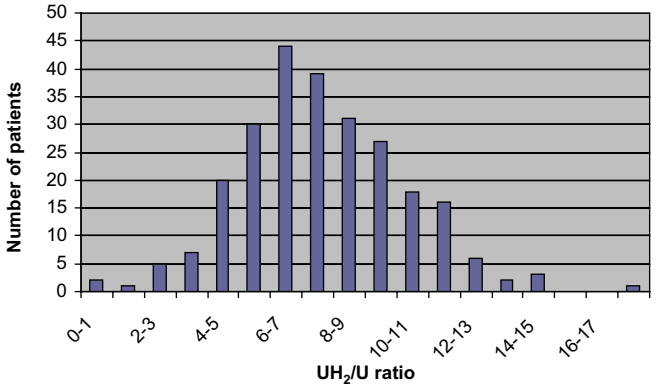

Fig. 2. Pretherapeutic distribution of UH_2/U ratios.

- h-1590T > C + h 85 T > C,
- h 2846A > T + h 85 T > C,
- h IVS14+1G > A + h −1590T > C,
- h IVS14+1G > A + h 2846A > T + h 85 T > C.

3.4. Correlation between phenotypic, genotypic parameters and clinical outcome

3.4.1. Correlation between 5-FU plasma clearance and toxicity

5-FU plasma clearance was compared between the 2 groups of patients, 1 and 2. A significant difference in 5-FU plasma clearance was found between the 2 groups in both regimens: 4 h/5-FU ($p < 0.001$) and LV5FU2 ($p = 0.026$), with a lower mean clearance for patients in group 2, with severe toxicity, compared to those in group 1: 40.2 *vs* 105 L h^{-1} m^{-2} for 4 h weekly 5-FU and 78 L h^{-1} m^{-2} *vs* 136.7 for LV5FU2.

3.4.2. Correlation between uracil concentration and both 5-FU plasma clearance and toxicity

A negative and significant correlation was found between uracil plasma concentrations and 5-FU plasma clearance with both protocols: 4 h weekly 5-FU ($r = -0.219$; $p = 0.05$) and LV5FU2 ($r = -0.221$; $p = 0.006$). The results are displayed on Fig. 3.

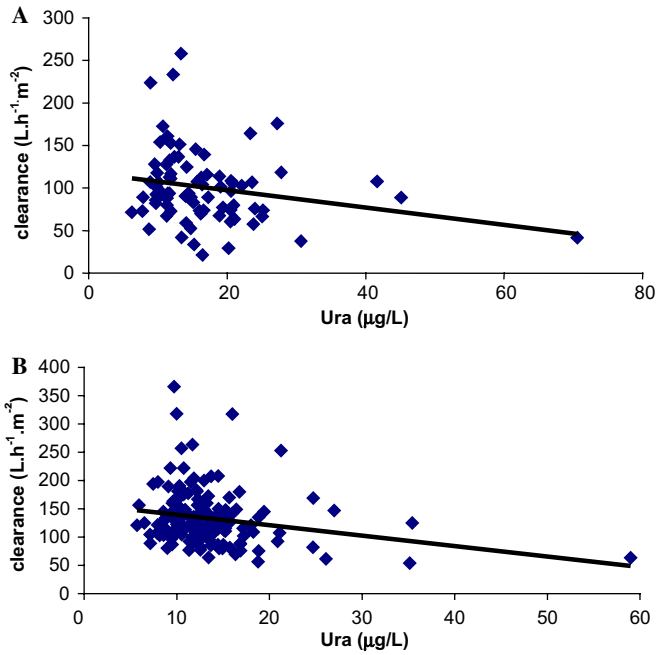

Fig. 3. Correlation between uracil plasma concentration (μg/L) and 5-FU plasma clearance (L h^{-1} m^{-2}) 4 h weekly 5-FU ($r = -0.219$; $p = 0.05$) (A) and LV5FU2 ($r = -0.221$; $p = 0.006$) (B).

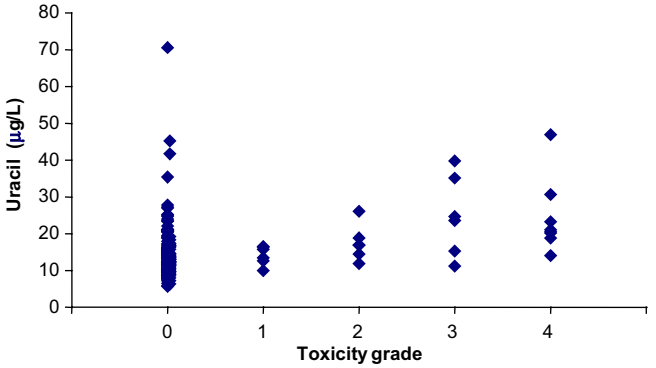

Fig. 4. Uracil plasma concentrations (µg/L) as a function of treatment grade of toxicity ($n = 234$).

A significant correlation was found between uracil plasma concentrations and 5-FU toxicity ($p < 0.001$) for 236 out of the 252 patients whose treatment-induced toxicity was known. The same analysis was performed without the two patients with very high uracil plasma levels. The correlation remained significant ($p < 0.001$). Mean uracil concentrations increased with toxicity grade: grade 0, 14 ± 6.7 µg/L; grade I, 14 ± 2.6 µg/L; grade II, 17.5 ± 4.8 µg/L; grade III, 25 ± 11 µg/L; grade IV, 24.4 ± 10 µg/L (Fig. 4).

We determined the uracil threshold value for toxicity based on an ROC curve: 15 µg/L.

3.4.3. Correlation study between UH_2/U ratio and 5-FU plasma clearance and treatment toxicity

No correlation was found between the UH_2/U ratio and 5-FU plasma clearance in both schedules, 4 h 5-FU/ ($p = 0.823$) or LV5FU2 ($p = 0.087$), when considering the whole range of UH_2/U ratio values. UH_2/U distribution function of 5-FU clearance is displayed on Fig. 5.

Actually, for high UH_2/U values, 5-FU plasma clearance remained quite unchanged and homogenous, rarely exceeding 100 L h^{-1} m^{-2} for 4 h 5-FU or 150 L h^{-1} m^{-2} for LV5FU2 regimen. We were especially interested in low UH_2/U ratios. Below 7.3, the median value, there was a significant positive correlation for the LV5FU2 regimen ($r = 0.301$; $p = 0.016$; $n = 63$), but not yet for 4 h 5-FU ($p = 0.191$; $n = 52$).

A significant correlation was found between UH_2/U ratio and treatment toxicity, as displayed on Fig. 6 ($p < 0.001$). The mean UH_2/U ratio decreased with toxicity grade: grade 0, 8 ± 2.5; grade I, 6.5 ± 3.2; grade II, 6.7 ± 1.7; grade III, 5 ± 3.1; grade IV, 3.7 ± 2.7.

The threshold value determined for severe toxicity based on ROC curve was 6.

3.4.4. Correlation between DPYD mRNA expression and 5-FU plasma clearance, uracil plasma concentration, UH_2/U ratio and toxicity

No relationship was found between mRNA expression and 5-FU plasma clearance ($p = 0.508$ and $p = 0.258$ for 4 h weekly 5-FU and LV5FU2), uracil concentration, ($p = 0.731$), UH_2/U ratio ($p = 0.173$) or toxicity ($p = 0.663$).

3.4.5. Influence of DPYD genotype upon 5-FU plasma clearance, toxicity, uracil concentration and UH_2/U ratio

The impact of the different SNPs on pharmacological and clinical parameters was evaluated and compared to those of patients without SNPs, i.e: (1) 5-FU plasma clearance: 104.7 ± 43.8 L h^{-1} m^{-2} (weekly 5-FU) and

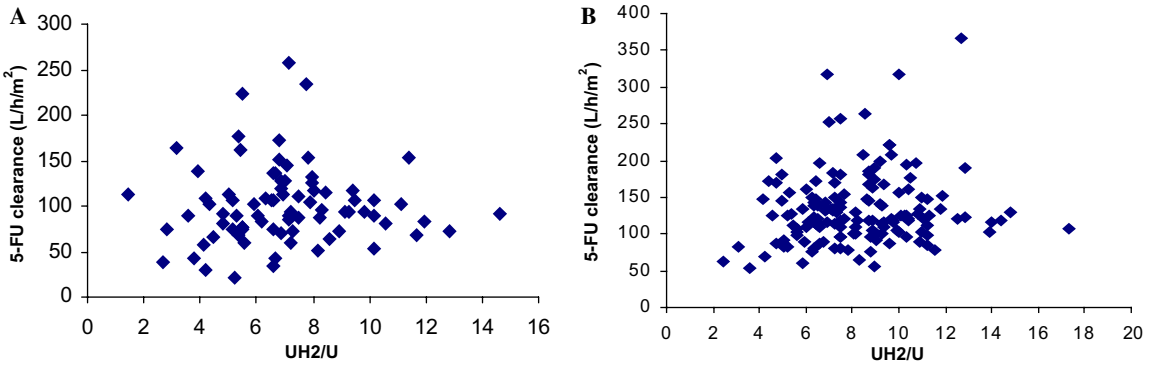

Fig. 5. Distribution of 5-FU plasma clearance (L h^{-1} m^{-2}) function of UH_2/U ratio for 4 h 5FU (A) and LV5FU2 (B).

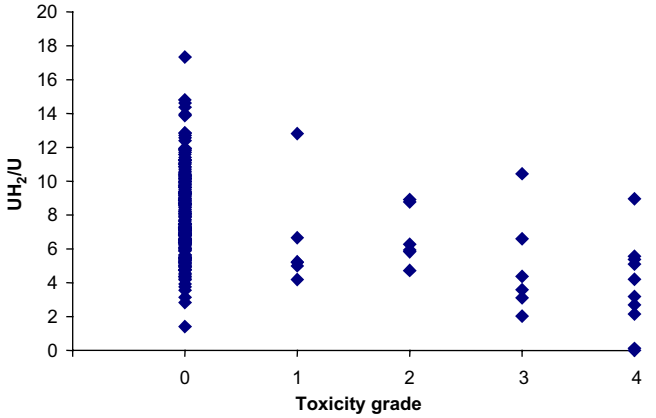

Fig. 6. UH_2/U plasma ratio function of the grading of toxicity.

Table 2
Statistical correlation between DPYD SNPs and 5-FU plasma clearance, uracil, UH_2/U ratio and severe toxicity, compared to patients with no SNPs

	Nb	5-FU Clearance ($L\ h^{-1}\ m^{-2}$) means ± SD		Uracil (μg/L) means ± SD	UH2/U means ± SD	% Toxicity grade III–IV
		LV5FU2	FuFol			
0 SNPs	163	136.0 ± 50.0	104.7 ± 43.8	13.9 ± 6.7	8.1 ± 2.5	5.6
85 T > C	79	130.6 ± 46.3	103.4 ± 38.2	16.0 ± 9.0	7.2 ± 2.6	6.33
		$p = 0.549$	$p = 0.895$	$p = 0.069$	$p = 0.758$	$p = 0.362$
−T1590C	3	133.7 ± 36.9	NA	16.9 ± 3.9	5.84 ± 0.27	0
		$p = 0.948$		$p = 0.557$	$p = 0.01$	$p = 0.708$
IVS14 + 1G > A	2	NA	21.22	18.8 ± 3.25	5.16 ± 0.07	50.0
			$p < 0.001$	$p = 0.36$	$p < 0.001$	$p < 0.001$
2846 A > T	7	81.2 ± 9.9	43.9 ± 14.6	22.0 ± 9.6	4.4 ± 1.6	71.4
		$p < 0.001$	$p = 0.002$	$p = 0.069$	$p = 0.001$	$p < 0.001$
2846 A > T + IVS14 + 1G > A	1	ND	NA	465	0.12	100

NA, not applicable.
ND, not determined.

$136 \pm 50\ L\ h^{-1}\ m^{-2}$ (LV5FU2); (2) plasma uracil, 13.9 ± 6.7 μg/L; (3) UH_2/U ratio, 8.1 ± 2.5; (4) severe toxicity at 2 first cycles, 5.6% of the patients (Table 2).

Several SNPs were detected but only 2 were of interest.

Three patients had IVS14 + 1 G > A variants; 2 had only this mutation, or also had another non-relevant variant, and 1 patient had both IVS14 + 1G > A and 2846 A > T. The first two patients had dramatically reduced 5-FU plasma clearance levels, close to 0 for one who experienced grade IV toxic side-effects after the first cycle, and $21.1\ L\ h^{-1}\ m^{-2}$ for the second one, who had grade I toxicity and then quickly a 50% 5-FU dose reduction ($p < 0.001$). Their UH_2/U ratios were 5.1 and 5.2, i.e. significantly reduced compared to those from patients with no SNPs ($p < 0.001$), however no significant difference was found for their uracil plasma levels.

2846A > T variant was detected in 8 patients, 7 times individually, and once combined with IVS14 + 1G > A.

For the 7 patients, this SNP led to a significantly decreased 5-FU plasma clearance level compared to patients with no variants: $43.9 \pm 14.6\ L\ h^{-1}\ m^{-2}$ for weekly 4 h 5-FU ($p = 0.002$) and $81.2 \pm 9.9\ L\ h^{-1}\ m^{-2}$ for LV5FU2 ($p < 0.001$).

Uracil plasma levels were higher too: mean: 22.0 ± 9.6 μg/L *vs* 13.9 ± 6.7 μg/L, but not significantly ($p = 0.069$), probably due to the small sample size. Likewise, UH_2/U ratios were significantly lower in patients with 2846A > T SNP: 4.4 ± 1.6 *vs* 8.1 ± 2.5; ($p = 0.001$).

Consequently, a significantly increased toxicity was observed, since 5 out of the 7 heterozygote patients had grade III–IV early toxic side-effects ($p < 0.001$). One patient had grade I toxicity directly following the first 5-FU administration; the 5-FU dose was quickly reduced. One of these patients had no toxic side-effects.

One patient had 3 heterozygote variants: IVS14 + 1G > A, 2846A > T and 85 T > C. He experienced grade

IV polyvisceral toxicity and died after 40 days in an intensive care ward. His 5-FU plasma clearance was close to 0, his UH_2/U ratio very low, 0.12, and his uracil plasma level very high, 465 ng/mL.

On the other hand, 85T > C variant, whether homo or heterozygote, clearly had no influence in terms of 5-FU plasma clearance, uracil concentration, UH_2/U ratio and toxicity (Table 2):

- Plasma clearance: 104.7 ± 43.8 L h^{-1} m^{-2} (no variant) *vs* 103.4 ± 38.2 L h^{-1} m^{-2} (h + Ho 85 T > C) weekly 4 h 5-FU ($p = 0.895$) and 135.9 ± 50.2 L h^{-1} m^{-2} (no variant) *vs* 130.6 ± 46.3 L h^{-1} m^{-2} (h + Ho) LV5FU2 regimen ($p = 0.549$),
- Uracil concentration: 13.9 ± 6.7 μg/L (no mutation) *vs* 16.0 ± 9.0 μg/L (h + Ho) ($p = 0.069$),
- UH_2/U ratio: 8.1 ± 2.5 (no mutation) *vs* 7.2 ± 2.6 (h + Ho) ($p = 0.758$),
- Toxicity: 5.6% (no mutation) *vs* 6.33% (h + Ho), ($p = 0.362$).

Then, for three patients who had 85 T > C variant plus 1 or 2 others, we took in account only the other ones.

Likewise, −1590 T > C variant was neither correlated with 5-FU clearance, ($p = 0.948$), toxicity ($p = 0.708$) nor uracil concentrations ($p = 0.557$) for the two patients pooled, 1 with −1590 T > C and 1 with −1590 T > C + 85 T > C. However, UH_2/U ratio was significantly reduced (5.6 *vs* 6) ($p = 0.01$). Since only two patients were concerned, a larger number is needed in order to provide useful data.

Taking in account these results, we considered IVS14 + 1G > A and 2846A > T as relevant SNPs and then being to be determined before treatment.

3.5. Comparison of the different tests

We characterized sensitivity, specificity, and positive and negative predictive values for each parameter and for their respective combinations and we compared them. The results are displayed on Table 3.

The concordance test, with the κ coefficient, showed that parameters gave independent results and that combining two parameters was better than only one, whatever it was, but that combining three was useless.

Actually, the best combination differed with the test: according to sensitivity and specificity tests, the best combinations were variant detection or uracil (88.2 and 69, respectively), mutation or UH_2/U ratio (88.2 and 78.4, respectively), and UH_2/U ratio and uracil (76.5 and 83.6, respectively). On the other hand, for the predictive positive value, the best ones were variant detection and uracil (0.8 and 0.9 for 2 different values of prevalence, 10% and 20%), mutation and UH_2/U ratio (0.72 and 0.85) and UH_2/U ratio or uracil (0.88 and 0.95).

However, these results did not take in account the time required for each technique and the time necessary for providing the results. In fact, we found that the best results were obtained with a two-step strategy: first, we combined variant detection and uracil plasma measurement because of their results for the predictive positive test, and also because both methods were quicker than UH_2/U ratio measurement; second, according to the first step results, in case of suspicion of a DPD deficiency, the UH_2/U ratio was determined, both to confirm the deficiency and to evaluate more precisely the residual level of activity and to determine an optimal initial 5-FU dosage. This algorithm provided 83% and 84 % sensitivity and specificity, respectively.

The decision tree is as follows:

1. In the case of no SNP being found and the uracil plasma level < 15 μg/L, the initial 5-FU dosage remains unchanged (65.2% of the patients)
2. In the case of:
   (a) The uracil plasma level ⩾ 15 μg/L, with no SNP, or
   (b) A heterozygote relevant SNP, with plasma uracil < 15 μg/L,
   (c) The uracil plasma level ⩾ 15 μg/L, with a heterozygote relevant SNP,the UH_2/U ratio is calculated in order to confirm whether or not DPD deficiency is

Table 3
Respective sensitivity (Se) and specificity (Sp), positive and negative predictive values (P.P.V., N.P.V.) for 2 different values of prevalence, 10% and 20% of the different parameters

Tests	Se (%)	Sp (%)	P.P.V.		N.P.V.	
			10%	20%	10%	20%
Relevant SNP	47.1	98.3	0.75	0.87	0.94	0.88
Uracil	88.2	69.4	0.24	0.42	0.98	0.96
UH_2/U ratio	82.4	78.4	0.30	0.49	0.98	0.95
SNP and uracil	47.1	98.7	0.80	0.90	0.94	0.88
SNP or uracil	88.2	69	0.24	0.42	0.98	0.96
SNP and UH_2/U ratio	41.2	98.3	0.73	0.86	0.94	0.87
SNP or UH_2/U ratio	88.2	78.4	0.31	0.51	0.98	0.96
UH_2/U ratio and uracil	76.5	83.6	0.34	0.54	0.97	0.93
UH_2/U ratio or uracil	94.1	64.2	0.89	0.95	0.99	0.98
3 Tests (and)	41.2	98.7	0.78	0.89	0.94	0.87
3 Tests (or)	94.1	64.2	0.23	0.40	0.99	0.98

present and hence to determine the 5-FU dose to be administered. If the UH_2/U ratio is

i. >6: The 5-FU dosage remains unchanged.
ii. Between 3 and 6: the 5-FU dosage is lowered to 50% compared to the standard dose and then an individual pharmacokinetic follow-up is proposed.
iii. Between 1.5 and 3: the 5-FU dosage is lowered to 70% compared to the standard dose and then an individual pharmacokinetic follow-up is proposed.
iv. <1.5: 5-FU administration is not recommended and an alternative treatment must be discussed. If 5-FU must be given, a dose reduction of at least 80% must be carried out with a close follow-up of 5-FU plasma levels within the hours following 5-FU administration.

Finally, in the case of a homozygous status for a relevant SNP, with a uracil plasma level higher than 100 μg/L or a UH_2/U ratio below 1, then fluoropyrimidine administration must be discussed and an alternative treatment proposed.

4. Discussion

In the present study, we compared different approaches for DPD deficiency detection prior to 5-FU-based therapy. Acute polyvisceral toxicity, after high and prolonged 5-FU plasma levels due to DPD deficiency, can occur as early as the first cycle of treatment with a frequency of about 3–5%; it is sometimes lethal (0.5%) [4,7–9]. Considering its frequent use, it would be important to detect patients with complete or partial deficiency prior to treatment, but no detection strategy and handling procedure has been proposed and accepted to date. Out of the 4 different approaches for detecting DPD deficiency, DPYD mRNA expression was not relevant. The 3 other ones, uracil plasma levels, UH_2/U ratio, and the detection of relevant DPYD SNPs, ($IVS14 + 1G > A$ and $2846A > T$) were highly correlated to toxicity and to the 5-FU plasma clearance at the first cycle in our population of 252 patients.

Thus, in our population of 118 patients, the enzyme activity seems to depend much more on variants of the DPYD gene, i.e. the functionality of the enzyme, than the levels of its mRNA expression. This is not surprising since a high level of mRNA cannot compensate an enzyme deficiency. However, we cannot conclude that the level of mRNA expression has no impact on enzyme activity, due to the too small number of patient.

Determination of uracil plasma levels by liquid chromatography was quite simple and rapid. Its threshold determined according to the ROC curve was 15 μg/L.

The UH_2/U ratio appeared less influenced by interfering parameters, but it required a longer specific chromatography method to perform the simultaneous measurement of both uracil and its first metabolite. Its threshold determined according to the ROC curve was 6.

DPYD SNP detection was quite interesting. It lasted 50 min for 1 SNP and 96 samples. Thus, results could be given within 10 days, making this technique highly suitable for clinical practice.

The 3 tests had different levels of sensitivity, specificity, and positive and negative predictive values. All of them were interesting but none was sufficient by itself for DPD deficiency detection. SNP detection was by far the most specific approach but was not sufficiently sensitive. On the contrary, uracil level determination in plasma was very sensitive but not sufficiently specific. Interestingly, uracil determination and the UH_2/U ratio did not provide redundant results and were independent of each other.

We tried to combine them and found that the best results, in terms of accuracy and speed were obtained with a two-step strategy: Firstly, SNP detection combined to uracil plasma measurement then, secondly, UH_2/U ratio determination to confirm a DPD deficiency and/or to more precisely determine the residual level of activity. This algorithm provided 83% and 84% sensitivity and specificity, respectively.

If this algorithm had been applied to our population of patients, an initial 5-FU dose reduction would have been decided for 98.8% of the patients who underwent grade 3–4 toxic side-effects in the first 2 cycles. On the other hand, less than 8% of the patients who had no severe toxic side-effects in the first 2 cycles would have had an initial 5-FU dose reduction. Furthermore, given the pharmacokinetic follow-up, these patients would have received an increased secondary dose and hence would not have been underdosed.

Finally, it must be pointed out that this protocol does not merely serve to detect DPD deficiency, but also serves for subsequent therapeutic advice. Except in cases where alternative treatment is recommended because the 5-FU metabolism is close to zero, $IVS14 + 1G > A$ or $2846A > T$ heterozygote, high uracil plasma levels or low UH_2/U ratios

are not strict contra-indications to 5-FU treatment, provided that the physician is aware of it and that added precautions are taken, 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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