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ORIGINAL ARTICLE

The influence of fluorouracil outcome parameters on tolerance and efficacy in patients with advanced colorectal cancer

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The purpose of this study was to determine simple genetic factors helpful to tailor 5-FU administration and determine strategy in first-line chemotherapy of advanced colorectal cancer. In 76 patients initially treated by 5-FU, thymidylate synthase, dihydropyrimidine dehydrogenase and methylene tetrahydrofolate reductase germinal polymorphisms, dihydrouracil/uracil plasma ratio and 5-FU plasma clearance were investigated and correlated for tolerance (10.5% grade 3 and 4 toxicity) and efficacy (32.9% objective response rate and 20 months median overall survival time). Toxicity was linked to performance status >2 ($P=0.004$), low UH_2/U ratio, 2846 A>T, IVS 14 + 1G>A for *DPD* ($P=0.031$), and homozygoty C/C for *MTHFR* 1298 A>C ($P=0.0018$). The overall survival of the patients with a 3R/3R *TS* genotype associated with C/C for 677 C>T or A/A for 1298 A>C was statistically shorter (log-rank test $P=0.0065$). Genetic factors permit the tailoring of 5-FU treatment. They should occupy center stage in future clinical trials for specifically designing treatment for patients with a given biologic feature.
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Keywords: colorectal cancer; fluorouracil; thymidylate synthase; dihydropyrimidine dehydrogenase; methylene tetrahydrofolate reductase

Introduction

Over the past few years, a central goal of research in oncology has been to determine main molecular biomarkers that could predict response and toxicity to chemotherapeutic agents.

Indeed, the understanding of interindividual heterogeneity in efficacy and toxicity could serve as a rational basis for tailored treatment (optimal drug therapy and dosage for each patient) as opposed to an empirical 'one-size-fits-everybody' chemotherapy. In this domain of a cancer pharmacogenetic approach, intense efforts have especially focused on fluorouracil and on its metabolic enzymes.

5-fluorouracil (5-FU) has been widely used for almost 50 years in the treatment of solid malignancies, especially in colorectal cancer (CRC) both in advanced and adjuvant settings.

Its main target is thymidylate synthase (TS), a key enzyme in cell proliferation, which is inhibited by the active metabolite, fluorodeoxyuridine monophosphate (FdUMP).

In patients with metastatic disease, response rates are below 20% with 5-FU monotherapy and go up to 25% simply by adding folinic acid, leucovorin (5-formyltetrahydrofolate), a precursor of the methyl donor 5–10 methylenetetrahydrofolate (CH_2FH_4).[1] Indeed, the complex between TS and FdUMP is stabilized in an inactive ternary form by CH_2FH_4.[2] Clinical studies have clearly demonstrated the interest of this association allowing improved TS inhibition.[3]

Despite the improved response rates with the addition of oxaliplatin or irinotecan, 5-FU remains a major drug in treatment of CRC.[4] However, many questions persist such as: how can we enhance the response rate and efficacy in terms of survival? How can we improve tolerance, knowing that treatment efficacy is correlated to the 5-FU dose, but dose-intensification strategies increase toxicity? Moreover, these toxic side effects, around 20–25% grade 3 and 4 events with current 5-FU regimens and 0.2% toxic deaths often occur in patients in adjuvant situations; many of them are either already cured by surgery before chemotherapy, 30% in pT3N1 and 70% in pT3N0 stages, or can receive no benefit from treatment.

To answer these questions, several new approaches are under investigation, such as pharmacogenomics, by analyzing tumor tissue, with microarrays, for example. The problem with pharmacogenomics is the generally high number of genes requiring bioinformatic study that does not make it technically suitable for routine work.

Another research approach is pharmacogenetics that focuses on the effects of polymorphisms (especially single-nucleotide polymorphisms (SNPs)) in drug-metabolizing enzymes and in drug targets. It could enable the discovery of the best-tolerated and most effective treatment strategies

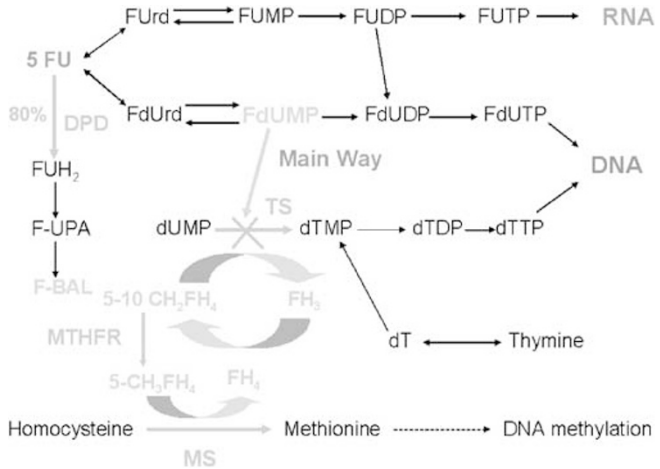

Figure 1 5-FU metabolic pathways. 5-FU, 5-fluorouracil; 5–10 CH_2FH_4, 5–10 methylenetetrahydrofolate; 5-CH_3FH_4, 5-methyltetrahydrofolate; FH_4, tetrahydrofolate; dUMP, deoxyuridine 5′-monophosphate; dTMP, deoxythymidine 5′-monophosphate; dTDP, deoxythymidine 5′-diphosphate; dTTP, deoxythymidine 5′-triphosphate; FdUMP, fluorodeoxythymidine 5′-monophosphate; FUR, fluorouracil; FUdR, 5-fluorodeoxyuridine; F-BAL, 5-fluoro-β-alanine; DPD, dihydropyrimidine dehydrogenase; TS, thymidylate synthase; MS, methionine synthetase.

by the study of the hereditary basis for interindividual differences in drug response. In our study, we decided to focus on three previously studied enzymes involved in fluorouracil metabolism and activity (Figure 1).

TS is the primary target. It catalyzes the reductive methylation of dUMP by CH_2FH_4 to form dNTP which is a critical reaction for cell proliferation. The *TS* gene on chromosome 18 contains a unique 28-bp tandem repeat sequence (called 'R') within the enhancer region (TSER) and within the 5′-untranslated region. The three most common genotypes in the Caucasian population (2R/2R, 2R/3R and 3R/3R) have been identified.[5] This tandem repeat sequence appears to function as an enhancer element on transcription as well as on translation of *TS* mRNA with an increasing number of repeat sequences.[6,7] Despite certain contradictory results, most clinical studies have documented the growing evidence that the *TS* genotype is associated with clinical efficacy in terms of response and survival to 5-FU-based chemotherapy.[8–11] Moreover, a common SNP has been recently identified at the 12th nucleotide of the second repeat in the 3R allele within a USF family E-Box consensus element. This SNP could abolish USF-1 binding and decrease *in vitro* transcriptional activity.[12,13] From these experimental observations, Kawakami *et al*.[7] classified each patient in 'Low' or 'High' *TS* expression in accordance with the combination of this SNP and TSER genotypic status (Table 1). Two clinical studies showed a correlation between this combination and overall survival (OS) (significantly longer in the low expression group).[12,14]

Dihydropyrimidine dehydrogenase (DPD) is the key enzyme involved in the catabolic breakdown of 5-FU. It is submitted to a common genetic polymorphism and its activity displays wide interindividual variability from 8- to 21-fold.[15–16] Low enzyme activity leads to excessive amounts of active metabolites and consequently to severe adverse events, hematopoietic, neurological and gastrointestinal, sometimes combined in a polyvisceral toxic syndrome, potentially fatal in 0.2% of cases. In the global population, 3% of individuals are heterozygous and at risk of adverse toxic events. To date, around 30 mutations on chromosome 1p associated with reduced DPD activity have been reported. For example, the most prominent *DPD* polymorphism is a G>A SNP in the GT 5′-splice recognition site of intron 14 that leads to skipping exon 14 and the synthesis of an inactive truncated protein with severe *DPD* deficiency.

Table 1 Classification following Kawakami *et al*.[7] in 'Low' or 'High' *TS* expression in accordance with the combination of SNP and TSER genotype status

'High' expression	*'Low' expression*
2R/2R	2R/3Rg
2R/3Rc	3Rc/3Rg
3R/3Rc	2Rg/3Rg

Abbreviations: CR, complete response; SNP, single-nucleotide polymorphism; TS, thymidylate synthase; TSER, thymidylate synthase enhancer region.

Methylene tetrahydrofolate reductase enzyme (MTHFR) controls the intracellular CH_2FH_4 concentration. It converts CH_2FH_4 into 5-methyltetrahydrofolate and represents a key enzyme in the folate metabolism at the crossroads between DNA synthesis and DNA methylation *via* methylation of homocysteine to methionine. *MTHFR* is also subject to several common polymorphisms on chromosome 1p. To date, 44 SNPs have been individualized. Among these, 37 are correlated with severe enzyme activity deficiency (inferior to 20%) and hyperhomocysteine. The *MTHFR* 677 C>T and 1298 A>C are the most common (15 and 12%, respectively, for homozygotes)[17] and homozygous mutated genotypes are associated with lower enzyme activity (a decrease of 75% for 677 C>T and 30% for 1298 A>C). However, an accumulation of intracellular CH_2FH_4 is observed which could enhance the efficacy of 5-FU treatment by stabilization of the ternary complex with TS and FdUMP. This concept was clearly demonstrated both in experimental studies and in clinical settings.[18–23]

The purpose of this study was to analyze the correlation between *TS*, *DPD* and *MTHFR* germinal polymorphisms on efficacy and toxicity of fluorouracil and leucovorin chemotherapy in patients with advanced CRC. Our objective was to provide a tool for physicians to determine before treatment patients at risk of 5-FU toxicity or resistance. To our knowledge, this study is the first that analyzes these three enzymes in a homogeneous population. We determined these different polymorphisms with usual biomolecular techniques, associated with the determination of fluorouracil clearance and a study of uracil and dihydrouracil (UH_2/U) plasma ratios as described previously.[24] The genetic polymorphism analysis was carried out from a simple peripheral blood sample that represents an interesting and easy non-invasive approach for molecular profiling. Finally, we evaluated the tolerance to and efficacy of fluorouracil treatment.

Results

Clinical data and frequencies of genotypes

A total of 76 patients who fulfilled the inclusion criteria were studied. The median follow-up of the study was 1264 days (3.5 years) with 7.7–85 months (7 years) as the range. The clinical data of these patients are provided in Table 2. The median age was 71-years old and 93.4% of patients had a good performance status, that is lower than 2. The sex ratio was 1.5/1 male/female. The site of metastasis was unique in 76% of the cases and then in 75% of cases in the liver. These clinical characteristics were in agreement with epidemiological data concerning advanced CRC.

In our study, 83% of patients were treated in first-line therapy.

Table 3 lists the distribution of *TS*, *DPD* and *MTHFR* gene polymorphisms. This distribution was similar with data previously described in a Caucasian population:[5,16,17,20] 18.4% for 2R/2R, 9.2% for *MTHFR* 1298 C/C, 10.6% for *MTHFR* 677 T/T, and 3.9% for *DPD* polymorphism heterozygotes. Moreover, these genotype frequencies were in agreement with the Hardy–Weinberg equilibrium and populations with different polymorphisms are homogeneous with respect to age, performance status and gender.

Table 2 Patients' initial characteristics

Clinical data	*Number of patients (%)*
Gender	
Male	46 (60.5)
Female	30 (39.5)
PS	
0–1	71 (93.5)
2–3	5(6.5)
Age	
Median (years)	71.2
Range (years)	39–88
Tumor site	
Colon	55 (72.5)
Rectum	21 (27.5)
Metastatic site	
Liver	44 (58)
Lung	4 (5.3)
Peritoneal	5 (6.5)
Carcinomatosis	
Nodes	5 (6.5)
Number of sites>1	18 (23.5)
Treatment regimen	
LV5FU2	31 (41)
Fufol 4 h	45 (59)
First line of treatment	63 (83)

Correlation between polymorphisms and toxicity

The different types of toxicity are described in Table 4. Sixty-six adverse events (87.9% grade 1 and 2 side effects) were notified, especially skin and gastrointestinal (47.5% in total): mucositis (7.5%), diarrhea (22%) and hand-foot syndrome (18%). Conjunctival irritation was not rare (17%) but often not much described in previous clinical data although it impaired the quality of life. The incidence of hematological events was low (only 3%). Finally, the incidence of cardiac toxicity was 6% but without constituted myocardial infarction.

With the tailored regimens using pharmacokinetic monitoring, the fluorouracil-based chemotherapy was well tolerated (only 10.5% grade 3 and 4 toxic side effects) and no toxic death was noted.

Among 76 patients of this prior study, nine patients (11.8% of the overall population) were isolated because of a high risk of fluorouracil toxicity. These nine patients presented abnormally low clearance levels of fluorouracil

Table 3 Distribution of *TS*, *DPD* and *MTHFR* polymorphisms in the studied population (76 patients)

	Number of patients	*Frequency (%)*
TS polymorphisms		
2R/2R	14	18.4
2R/3R	39	51.3
3R/3R	23	30.3
TS expression		
'High'	50	65.8
'Low'	26	34.2
MTHFR 1298 A>C		
A/A	40	52.6
A/C	29	38.2
C/C	7	9.2
MTHFR 677 C>T		
C/C	32	42.1
C/T	36	47.3
T/T	8	10.6
DPD		
2846 A>T	2	2.6
IVS 14 + 1G>A	1	1.3

Abbreviations: DPD, dihydropyrimidine dehydrogenase; MTHFR, methylene tetrahydrofolate reductase; TS, thymidylate synthase.

Table 4 Distribution of the toxic side effects in the population of patients (76 patients)

	Grade 1	Grade 2	*Grade 3*	*Grade 4*	*Total* (n)
Mucositis	2	2	0	1	5
Conjunctivitis	4	7	0	0	11
Diarrhea	5	8	2	2	17
Hand-foot syndrome	5	7	0	0	12
Cardiac	0	3	1	0	4
Hematologic	0	1	1	0	2
Nausea	2	3	0	0	5
Alopecia	0	1	0	0	1
Asthenia	4	4	1	0	9
Total (*n*)	22	36	5	3	66

associated with an abnormal UH_2/U plasma ratio: two patients were heterozygous A/T for 2846 A>T, one for IVS 14+1G>A and six without *DPD* SNP (epigenetic factors or SNP still unknown). In this population, nine toxic events were described, with 33.3% (and up to 66% for DPD heterozygoty) grade 3 and 4 gastrointestinal events, in spite of pharmacological adjustment and clinical observation. Concerning toxicity, a significant difference ($P=0.048$, Fisher's test) was found between the populations with (nine patients) and without a risk factor (67 patients).

Multivariate analysis of clinical and biological parameters for predicting toxicity according to the Cox-regression model is shown in Table 5. Fluorouracil toxicity was significantly linked to performance status >2 with 60% grade 3 and 4 events ($P=0.004$), low clearance and heterozygoty for *DPD* ($P=0.031$), and homozygoty C/C for *MTHFR* 1298 A>C ($P=0.0018$) with 30% grade 3 and 4 toxic events.

Table 5 Multivariate analysis of clinical and biological parameters for predicting toxicity according to the Cox-regression model

	Odds ratio (95% CI)	P
Performance status 2–3	*57.74 (3.70 ; 901.25)*	*0.004*
2846 A>T, IVS14+1G> A low clearance of 5-FU	*6.20 (1.18; 32.56)*	*0.031*
Age>75 years	0.08 (0.005; 1.09)	0.058
MTHFR 1298 A>C		
A/A	1.00 (ref)	0.056
A/C	2.54 (0.30; 21.47)	0.393
C/C	*25.99 (1.76; 384.32)*	*0.018*

Abbreviations: CI, confidence interval; MTHFR, methylene tetrahydrofolate reductase.

Correlation between polymorphisms and response

The global objective response (OR) (complete response plus partial response (PR)) rate was 32.9 with 6.6% of complete response (CR).

We considered the 'high risk for toxicity' patients as a possible bias for the efficacy study (because of inadequate dosage, toxic side effects leading to delayed treatment, bias between *DPD* SNPs and *TS/MTHFR* SNPs for interpretation of results). Consequently, the following studies (correlation between genotypes and response or survival) were analyzed on 67 patients after the exclusion of the nine with 5-FU low clearance and there was no difference for response rates (34.3% for OR and 7.4% for CR) in this new studied population.

The distribution between response rates and the different genotypes is shown in Table 6. No significant link was noted. However, the OR for 3R/3R was decreased by almost 50% in comparison with other TSER genotypes, as described in previous clinical studies. OR in the 'High TS expression' population also decreased with only 24 against 40.5% in the 'Low' group (not significant). Likewise, no variable was significant in multivariate analysis of biological parameters for predicting response to fluorouracil treatment.

Correlation between polymorphisms and survival

Among 67 patients, the OS rate was 5% at 5 years (Figure 2) in agreement with clinical data from the literature. The median OS time was 20 months. The median progression-free survival (PFS) time was 100 days. The log-rank analysis according to clinical characteristics was significant, with a longer survival for performance status <2 ($P=0.03$) and an age younger than 75-years old ($P=0.034$).

Table 6 Comparison of responses between the different genotypes in population without risk factors (67 patients)

	Objective response (%)	*Stability/ progression (%)*
Polymorphism TS		
2R/2R ($n=11$)	36.4 ($n=4$) CR = 18, 2 ($n=2$)	63.6 ($n=7$)
2R/3R ($n=35$)	42.9 ($n=15$) CR = 8, 6 ($n=3$)	57.1 ($n=20$)
3R/3R ($n=21$)	19.0 ($n=4$) CR = 0 ($n=0$)	81.0 ($n=17$)
Expression TS		
'Low' ($n=42$)	40.5 ($n=17$) CR = 11, 9 ($n=5$)	59.5 ($n=25$)
'High' ($n=25$)	24.0 ($n=6$) CR = 0 ($n=0$)	76.0 ($n=19$)
MTHFR 677 C>T		
C/C ($n=28$)	46.4 ($n=13$) CR = 10, 7 ($n=3$)	53.6 ($n=15$)
C/T ($n=31$)	22.3 ($n=7$) CR = 6, 5 ($n=2$)	77.4 ($n=24$)
T/T ($n=8$)	37.5 ($n=3$) CR = 0 ($n=0$)	62.5 ($n=5$)
MTHFR 1298 A>C		
A/A ($n=37$)	37.8 ($n=14$) CR = 8, 1 ($n=3$)	62.2 ($n=23$)
A/C ($n=25$)	32.0 ($n=8$) CR = 8, 0 ($n=2$)	68.0 ($n=17$)
C/C ($n=5$)	20.0 ($n=1$) CR = 0 ($n=0$)	80.0 ($n=4$)

Abbreviations: CR, complete response; *MTHFR*, methylene tetrahydrofolate reductase; *TS*, thymidylate synthase.

Univariate analysis for OS time according to TSER genotypes was not significant and curves crossed (Figure 3). However, OS medians were very different among TSER polymorphisms: 669, 900 and 270 days for 2R/2R, 2R/3R and 3R/3R, respectively. The log-rank test was highly significant after gathering 2R/3R and 2R/2R versus 3R/3R ($P=0.0066$).

No difference was found between OS time and 'High' or 'Low' TS expression with two superposable curves (Figure 4). Likewise, there was no difference between OS or disease-free progression survival times and the studied *MTHFR* genotypes (677 C>T and 1298 A>C).

However, the OS rate of the patients with the 3R/3R TS genotype associated with C/C for 677C>T or A/A for 1298A>C (16 patients (23.8%)) was much statistically shorter than that of the patients with another genotype (log-rank test $P=0.0065$) (Figure 5). The OS medians were 283 (95% CI (163; 603)) and 842 days (95% CI (515; 984)), respectively. There was no statistical difference between these populations of 16 patients and the others in respect to age, performance status and gender.

No variable, except age ($P=0.10$ if older than 75-years old, but with a possible bias such as death by another cause than cancer) or performance status ($P=0.0008$ if $\geqslant 2$), was significant in uni- or multivariate analysis for OS or PFS according to the Cox-regression method.

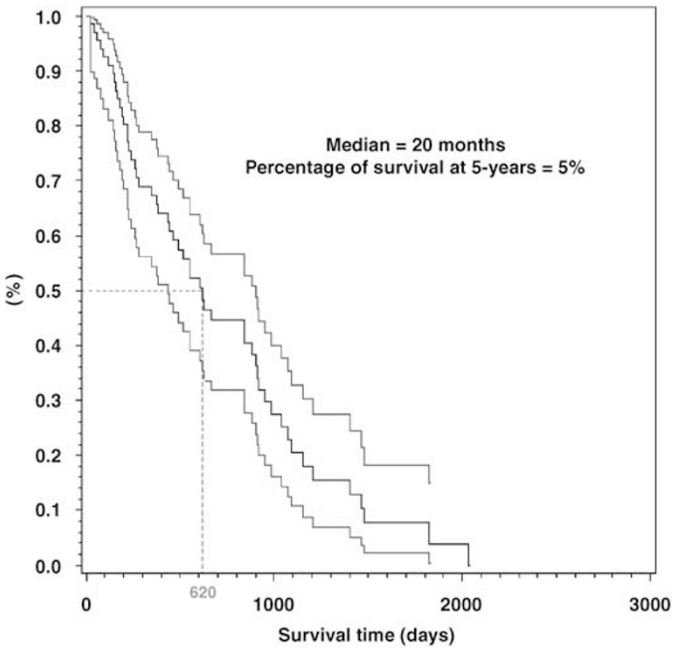

Figure 2 Kaplan–Meier curve with confidence interval (CI) at 95% for overall survival (76 patients).

Discussion

New targeted drugs have transformed the treatment of advanced CRC but the median OS is about 24 months in the absence of metastasis surgery, and 5-FU remains the cornerstone of the combinations. Furthermore, after previous clinical trials that showed an improved survival with combinations, recent multicentric randomized clinical trials comparing one versus two drugs in first-line therapy presented in abstract form showed no substantial difference in OS and treatment duration in the combined group, and on the contrary a more important toxicity.[25-28] Clearly, these recent results pose both the problem of the choice of the drugs and that of their combination. Thanks to tremendous progress in molecular biology, several promising genomic or genetic prognostic factors have been reported and could be useful but treatment strategies are still based on large clinical trials and translational studies are still ancillary. The purpose of this study was to simultaneously characterize the impact of pretherapeutically determined genetic and epigenetic factors on 5-FU tolerance and efficacy and based on the relevant factors to provide physicians with some tools to decide between one drug versus combined therapy in first line and to tailor 5-FU treatment.

To our knowledge, this is the first study analyzing the impact of both TS, DPD and MTHFR, the three main enzymes of 5-FU activity and metabolism, on its tolerance

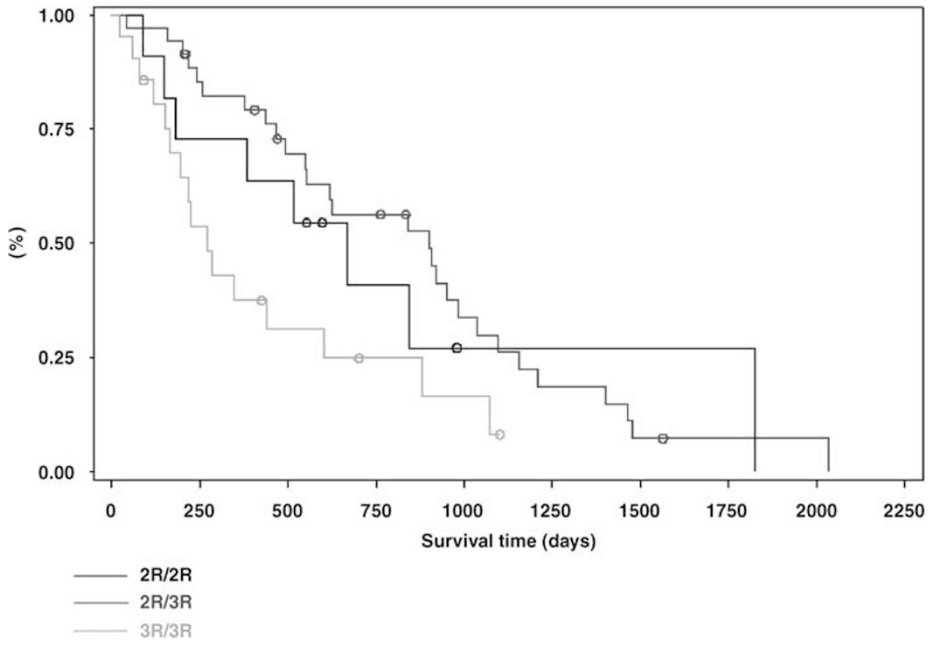

Figure 3 Univariate overall survival of patients according to TSER genotypes.

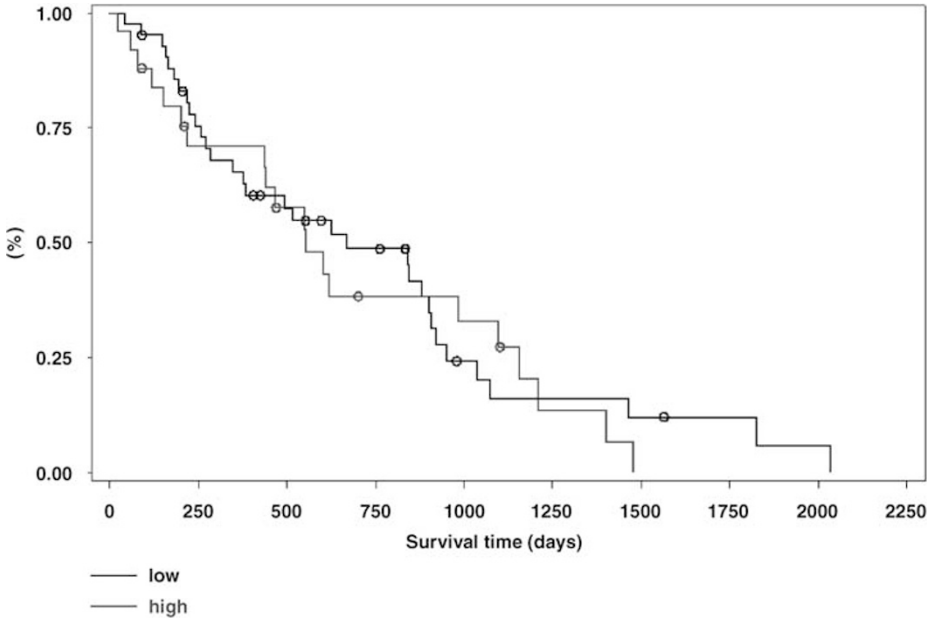

Figure 4 Univariate overall survival of patients according to low and high TS expression genotype. TS, thymidylate synthase.

and its efficacy in a homogeneous population of patients treated with a 5-FU and leucovorin combination for advanced CRC.

Indeed 5-FU, the most commonly used anticancer agents, provoke 20–25% grade 3 and 4 toxic side effects and 0.2–0.5% toxic deaths to patients under treatment while many of them are in an adjuvant setting, especially for colon cancer, and potentially cured after surgery.[4] Individual 5-FU dose management based on a pharmacokinetic follow-up permits us to reach the best therapeutic index but it does not prevent very early acute toxic side effects due to *DPD* deficiency and chronic toxicities such as dacryocystis, mainly due to *TS* polymorphism. Thus, in this study, the two regimens of treatment using pharmacokinetic monitoring as previously described were well tolerated in an elderly population with a median age of 71 years. However, 10.5% of grade 3 and 4 toxic events were observed. Concerning the metabolic pathway of 5-FU, we found that with the

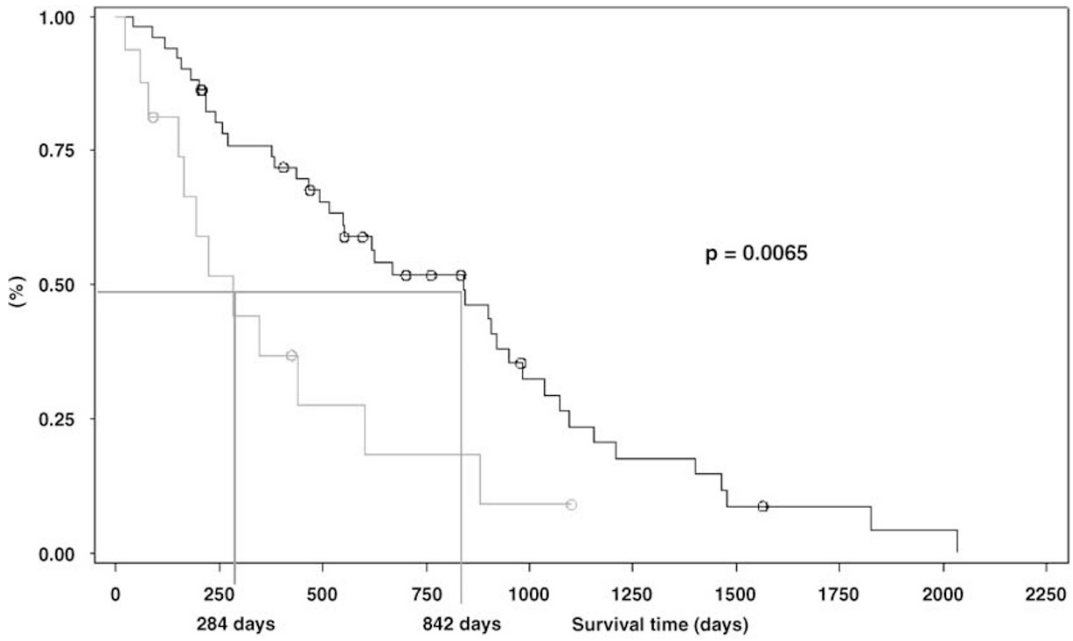

Figure 5 Univariate overall survival of patients according to 3R/3R for TS and A/A for MTHFR 1298 A>C or 3R/3R for TS and C/C for MTHFR 677 C>T genotypes (16/67 patients = 23.9%) (gray line) and other genotypes (black line). MTHFR, methylene tetrahydrofolate reductase; TS, thymidylate synthase.

pretherapeutic detection, 9 of the 76 patients were at high risk of 5-FU toxicity because of a DPD deficiency, revealed by a very low pretherapeutic UH_2/U plasma ratio.[24] Two of them were heterozygous A/T for 2846 A>T, one for IVS 14 + 1G>A, the main deleterious variants of the DPD gene. The six other patients had no *DPD* variant detected in the 23 that were analyzed. In these nine patients, nine very early toxic events were described, with 33.3% grade 3 and 4 gastrointestinal events in spite of pharmacological adjustment and clinical observation. This percentage of grade 3 and 4 adverse events reached 66% for the three patients with *DPD* variants. Thus, for early toxicity, a significant difference was found between populations with and without metabolic risk factors. Clearly, a pretherapeutic detection of DPD deficiency would have permitted the avoidance of these adverse events with an initial reduction of the 5-FU dose followed by individual dose management. Besides, some patients had an accelerated 5-FU metabolism due to an enhanced DPD activity that follows a Gaussian curve in the population.[15] These patients would benefit from intensified doses of 5-FU.

Likewise, patients whose *MTHFR* 1298 A>C genotype was C/C were significantly at higher risk of toxicity as well ($P = 0.018$).

On the other hand we focused on efficacy. The OR rate was 32.9%, with 6.6% of complete responses in monotherapy, and the median OS and PFS were 20 and 3.3 months, respectively.

In terms of response, we found that the OR rate for 3R/3R was decreased by almost 50% in comparison with other TSER genotypes, as described in previous clinical studies. Likewise, although statistically not significant, the OR rate was 24% versus 40.5% in 'High' compared with 'Low' *TS* expression.

We found no difference between OS and *TS* expression in contradiction with previously described results[12,14] but interestingly we found that an OS rate of 28% for the whole population of patients, sharing 3R/3R *TS* genotype associated with C/C for 677 C>T or A/A for 1298 A>C was significantly far shorter than that of patients with another genotype, regardless of its kind ($P = 0.0065$). Figure 5 shows the striking difference between the two curves, with a median survival rate of 283 versus 842 days, respectively. The other major interest of this finding is that clearly the OS rate of this population of patients remained poor despite the second-line combined therapy with oxaliplatin or irinotecan. Thus, an initial intensified first-line therapy, with a targeted therapy would probably have been of interest.

These results, both in terms of tolerance and efficacy, have a crucial impact on clinical practice. First, indeed, from a simple blood sample taken before chemotherapy, physicians can be warned of a high risk of severe or even life-threatening toxicity for a patient, that is relevant *DPD* and/or *MTHFR* variants regardless of the 5-FU regimens and the type of cancer.[23,29] Taking into account the wide use of 5-FU and other fluoropyrimidines, this is of major interest. In practice, in cases of a DPD deficiency, either 5-FU dose management based on a pharmacokinetic follow-up can be proposed after a reduced first dose of 5-FU or the switch to another drug, such as raltitrexed.[29] In the case of *MTHFR* 1298 A>C homozygote variant, the toxicity is not due to a metabolic deficiency. Then a 5-FU dose reduction could be useful but might impact the efficacy. Thus, a much closer

follow-up will be proposed and a dose reduction at the very beginning of a relevant adverse event.

Besides, the question of the one or two even three drugs in first-line therapy remains unresolved, except in case of selected patients, such as those with potentially resectable or curable liver and/or lung metastatic disease. For the other ones, who represent the majority of the patients, intensification has not clearly proved its superiority in terms of OS and has shown an increased frequency of toxic side effects.[27,28] Pharmacognenomics and pharmacogenetics could help the clinician to decide whether or not he has to intensify the chemotherapy regimen. As such, the detection of 3R/3R *TS* genotype associated with C/C for 677 C>T or A/A for 1298 A>C, is a major predictive factor of failure to 5-FU and of a very short survival rate. It obviously requires an immediately intensified combination with another cytotoxic drug such as oxaliplatin or irinotecan and a targeted therapy, such as an anti-VEGF or an anti-EGFR.[8,14,18–22] Intensification using one or two additional drugs with different metabolism pathways could restore the efficacy. Marcuello *et al*.[14] suggested that a combined regimen with irinotecan or oxaliplatin could reduce the predictive value of the *MTHFR* genotype in contradiction to other previous studies with 5-FU alone.[19,20,21,23] No results have been reported with both *TS* and *MTHFR* genotypes.

On the contrary, the other *TS* and *MTHFR* genotypes are predictive of very long survival with 5-FU plus folinic acid alone, and pose the question of the interest of intensified and combined protocols. Actually, except for some patients who could benefit from early metastasis surgery and need an immediate intensified chemotherapy, the question remains of the initial combination and the risk of toxic side effects. This is of major importance in the adjuvant setting. Undoubtedly, the FOLFOX regimen has statistically improved the cure rate compared with LV5FU2 in patients with pT3N1 colon cancer.[30] However, oxaliplatin provokes severe chronic neuropathy in 18–20% of patients that leads to a prolonged and sometimes irreversible functional impairment and alters the quality of life. Moreover, this protocol is now extended to pT3N0 patients whose risk of recurrence is 30%. Obviously, some patients would benefit from 5-FU plus folinic acid only and the screening for 3R/3R *TS* genotype associated with C/C for 677 C>T or A/A for 1298 A>C could help to detect patients free of these poor prognosis molecular factors.

Over the past few years, intensive efforts have focused on the problem of prognostic factors, both in terms of tolerance and efficacy. Our results confirm the interest for the detection of genetic factors such as *TS*, *DPD* or *MTHFR* polymorphisms and their potential impact in clinical practice. We have shown that this adjustment can be performed routinely using both simple biomolecular techniques and pharmacokinetic monitoring and therefore these factors should be used in future trials for tailoring therapy and design specifically the treatments for patients with a given biologic feature.

Materials and methods

Patients

This retrospective study included 76 patients treated for advanced CRC with two different regimens, weekly or every 2 weeks, but including only fluorouracil and leucovorin. To be eligible for inclusion, patients had to be over 18-years old, present measurable metastatic lesions, and have a life expectancy of at least 3 months. It was a first line of treatment for 83% of patients, and a second or more line of treatment for 17%. All patients were required to have normal bone marrow and organ functions (particularly cardiac function) before the administration of fluorouracil. Performance status was evaluated as defined by the World Health Organization (WHO). Written informed consent was obtained from all patients before taking peripheral blood samples for biomolecular analysis. A computed tomography scan was performed before the beginning of treatment and metastatic lesions were measured.

Chemotherapy regimen description

Two regimens of 5-FU plus leucovorin were administered, either by a weekly 4-h 5-FU continuous infusion (FUFOL 4 h) through a battery-operated pump after 100 mg/m^2 intravenous bolus of leucovorin, or every 2 weeks 46-h so-called LV5FU2 ('de Gramont' tailored regimen) after 200 mg/m^2 intravenous bolus of leucovorin and 400 mg/m^2 bolus of 5-FU.[4,31] The initial dose was 1200 or 2500 mg/m^2, respectively. Previous studies showed a significant relationship between 5-FU area under curve (AUC) in plasma and on one hand tolerance and on the other hand efficacy and a targeted AUC of 25 mg h l^{-1} had be determined, suitable to different kinds of 5-FU infusions.[32] Thus, 5-FU dose was tailored to reach this AUC using pharmacokinetic monitoring as described previously.[33] Briefly, 5-FU individual dose management was performed weekly or bi-weekly based on 5-FU steady-state plasma concentrations measured at the previous administration, at 3 h for weekly 5-FU or 43 h infusion for LV5FU2 and guided by a dose-adjustment chart.[32] Treatment was continued until progression was documented. Then, a second-line therapy combining 5-FU to oxaliplatin or irinotecan was proposed.

Follow-up

Every week or every 2 weeks, a physical examination was performed and toxic adverse events were evaluated and graded. Treatment efficacy was evaluated by comparing metastatic lesion measurements before and after 3months then every semester. Data were collected until the death of the patient or until the study's date point (1 December 2004).

Assessment of response

The response was assessed according to Response Evaluation Criteria in Solid Tumors Group Criteria and in reviewing computed tomography scans. The OS was defined by the period between the date of diagnosis and the date of death regardless of the cause or point date. PFS was defined by the

period between the date of diagnosis and the date of progression (clinical, biological or radiological progression) or death.

Assessment of tolerance

All adverse events, especially gastrointestinal events, mucositis, hand-foot syndrome, conjunctival irritation, and leukopenia were recorded and graded for severity according to WHO scales. Hemograms were performed every week, and ionograms, urea, creatinine and liver enzyme tests every 2 weeks.

In the event of significant grade 2 toxicity, and without pharmacokinetic recommendations for a decrease, the dose was reduced by 10%. In cases of grade 3 toxicity, the treatment was interrupted until toxic manifestations were resolved and restarted with a decrease of 25%. Treatment was stopped in cases of grade 4 toxicity.

Fluorouracil clearance

5-FU clearance was systematically calculated for each patient, especially since a low clearance can possibly be linked to unknown epigenetic factors that enhance the risk of toxicity.

This clearance was calculated by the following pharmacological formula:

$$\text{Fluorouracil clearance } [(l\times h)/\text{m}^2] = \frac{\text{Continuous infusion speed } [(\text{mg}\times h)/\text{m}^2]}{\text{Steady-state FU concentration } [\text{mg/l}]}$$

with continuous infusion speed = 5-FU dose (mg/m^2)/ infusion period (h). Thus 5-FU clearance for 'tailored LV5FU2' regimen was 132.84 ± 43.37 L × h/m^2 and 5-FU clearance for 'FUFOL 4h' regimen was 103.4 ± 51.52 L × h/m^2. Using the reduced difference distribution method, patients with FU clearance below 89.47 (l × h/m^2) (132.84 ± 43.37) for 'tailored LV5FU2' and inferior to 52.22 (l × h/m^2) (103.4 ± 51.52) for 'FUFOL 4 h' were considered as having a high risk of toxicity.

Genotyping

Genomic DNA was extracted from peripheral leukocytes by the salting-out procedure.[34]

Determination of TSER polymorphism. For the analysis of the 28-repeat polymorphism, a fragment containing the repeats was amplified using the two following primers: Forward primer was determined after research on Primer 3 Output© -CGCGGAAGGGGTCCTGC- and reverse primer as previously described by Kawakami *et al.*[7] -TCCGAGCCGGCC ACAGGCAT-.

Expected fragment sizes were 108 bp for 2R and 136 pb for 3R. In each case, PCR tests were run on HYBAID in a 50 μl final volume containing 1 μl of genomic DNA (100 ng/μl), 5 μl buffer 10 ×, 3 μl MgCl2 25 mM, 2 μl dNTPs, 0.4 μl Taq Polymerase, 2.5 μl DMSO (for final 5% volume), 1 μl of each specific forward and reverse primer (10 μM) and 34.1 μl H_2O.

After 35 cycles of amplification (denaturation at 94°C for 30 s, annealing at 62°C for 60 s, and extension at 72°C for 1 min), amplification products were electrophoresed in 1 × TBE on agarose gel at 2% with ethidium bromide 1 × (0.5 μg/μl) (Figure 6).[12–14,21]

Determination of G > C SNP. For the determination of G > C SNP within the second repeat in the 3R allele, restriction-fragment length polymorphism analysis was used. Briefly, amplification products for 3R/3R or 2R/3R genotypes were purified using the QIAquick system kit (Qiagen, Courtabeeuf, France) using the selective binding properties of a silica-gel membrane. According to provided buffers, impurities were washed away and the pure DNA was eluted during centrifugation (13 000 tr/min). Purified products (30 μl) were digested with the *Hae*III (Sigma, Lyon, France) restriction enzyme (GGCC restriction site) for 150 min in the presence of buffer 2 (4 μl for a final concentration 1 ×). Finally, digested PCR products were loaded into adjacent lanes on a 3% agarose gel containing ethidium bromide 1 × and electrophoresed in 1 × TBE (Figure 7).[12–14,21]

Determination of DPD and MTHFR polymorphisms. The analysis of *DPD* and *MTHFR* polymorphisms was based on pyrosequencing technology (bioluminometric real-time sequence determination).

For DPD polymorphisms, four SNPs were systematically analyzed: IVS 14 + 1G > A within intron 14, 2846 A > T within exon 22, 1679 T > G within exon 13 and 464 T > A

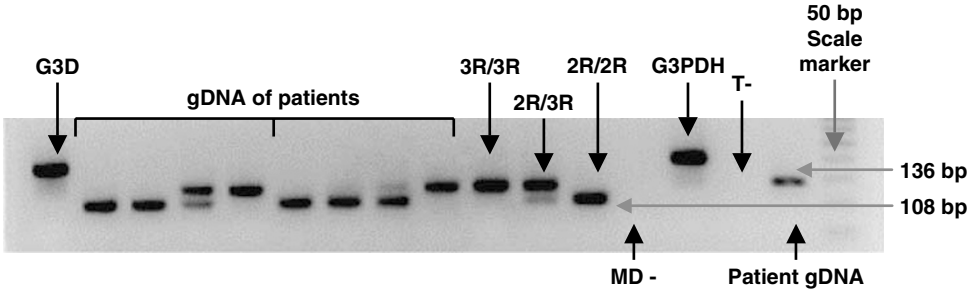

Figure 6 Thymidylate synthase enhancer region (TSER) analysis by PCR. Products can be separated in 1 × TBE on 2% gel electrophoresis with ethidium bromide 1 × (0.5 μg/μl). Sizes of different fragments are indicated on the right (fragments of 136 bases pairs (bp) and 108 bp corresponding to 3R and 2R, respectively). *G3PDH* = glyceraldehyde-3-phosphodehydrogenase (gene constitutively expressed in cells). G3D = G3PDH with dimethylsulfoxide (DMSO). MD− = negative marker with DMSO.

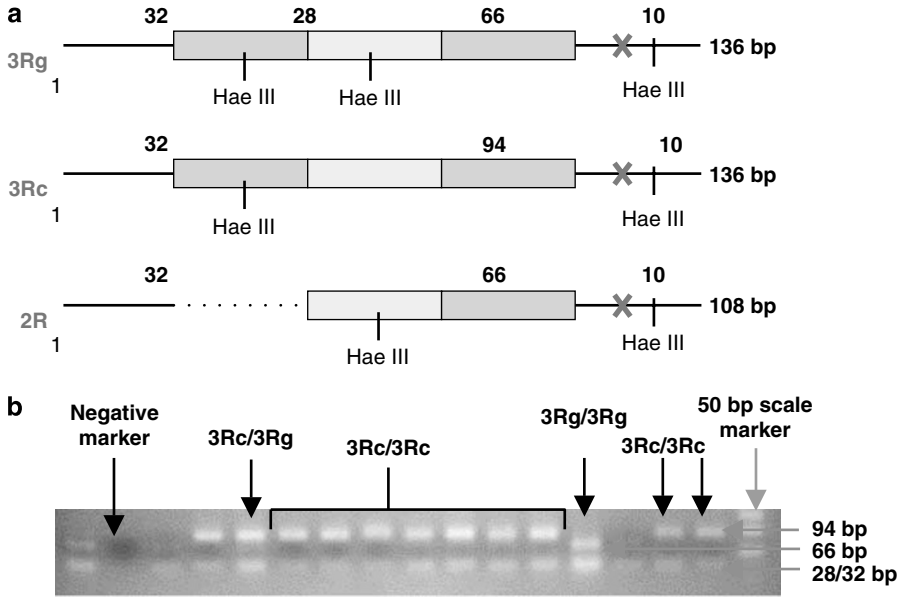

Figure 7 *Hae*III restriction map of TS tandem repeat fragments produced in the RFLP analysis. (**a**) This map shows the *Hae*III restriction sites within the fragments produced by PCR. ATG codon is indicated by the red cross. Theorical different sizes (bases pairs) of the DNA fragments produced after digestion with *Hae*III are shown. An *Hae*III restriction site within the second repeat of the 3R allele is removed with G →C SNP. Thus digestion allows for screening of this SNP. (**b**) Finally, digested PCR products were loaded into adjacent lanes on a 3% agarose gel containing ethidium bromide 1 × and electrophoresed in 1 × TBE. For each patient, the genotype was analyzed. RFLP, restriction-fragment length polymorphism; TS, thymidylate synthase.

Table 7 ***DPD*** **and** ***MTHFR*** **pyrosequencing. Primers used for biotinyled PCR and annealed primers used for bioluminometric real-time sequence determination**

	SNPs	*Exon*	*PCR primers 5′ → 3′*		*Annealed primers*	*Size (pb)*
			Forward	*Biotinyled reverse*		
DPD	IVS14+1G>A	Intron 14	atcagtgagaaaacggctgc	taaacattcaccaacttatgcca	aggctgactttccaga	150
	464 T>A	5	ttatggagctgctaagatga	atcatacatacctcagtagcaaa	cccattaatattggt	200
	1679 T>G	13	aatatggagcttccgtttct	gagagaaagttttggtgagg	ccagccaccagcacatcaa	204
	2846 A>T	22	aagcactgcagtaccttggaa	tcatgtagcatttaccacagttga	gcaagttgtggctatga	107
MTHFR	677 C>T	4	tattggcaggttaccccaaa	ctcacctggatgggaaagat	agaaggtgtctgcgg	208
	1298 A>C	7	gccaggggcaattcctctt	cttccagcatcactcactttgt	ggagctgaccagtgaag	202

Abbreviations: CR, complete response; DPD, dihydropyrimidine dehydrogenase; MTHFR, methylene tetrahydrofolate reductase enzyme; SNP, single-nucleotide polymorphism.

within exon 5, a nonsense mutation recently individualized in our research unit.[35,36]

These SNPs are associated with severe DPD deficiency and a potential life-threatening toxicity under a 5-FU regimen.

For MTHFR polymorphisms, the two most common SNPs in the Caucasian population were analyzed: 677 C>T and 1298 A>C.[20–23]

For each polymorphism, a prior biotinyled PCR with specific primers (Table 7) was performed. Briefly, DNA simple strands were obtained by the purification of the biotinyled PCR products with Sepharose balls coupled to streptavidine and after denaturation with washing in different baths (ethanol 70%, then NaOH 0.2 M and finally washing buffer pH 7.6 which washed away unbiotinyled products). These purified DNA simple strands were the target templates for sequencing by synthesis. Indeed the sequential addition of nucleotides (dNTP) into the growing DNA chain from an annealed primer was complementary to these target templates and each incorporation of dNTP by DNA polymerase led to the release of a pyrophosphate (Ppi). This Ppi was converted by ATP sulfurylase into ATP serving as a substrate for Luciferase enzyme. The produced light was detected as evidence of nucleotide incorporation and was translated as an on-screen pyrogram. Pyrosequencing data (pyrogram) for each patient were then compared to theoretical patterns and the genotype was easily identified (Figure 8).

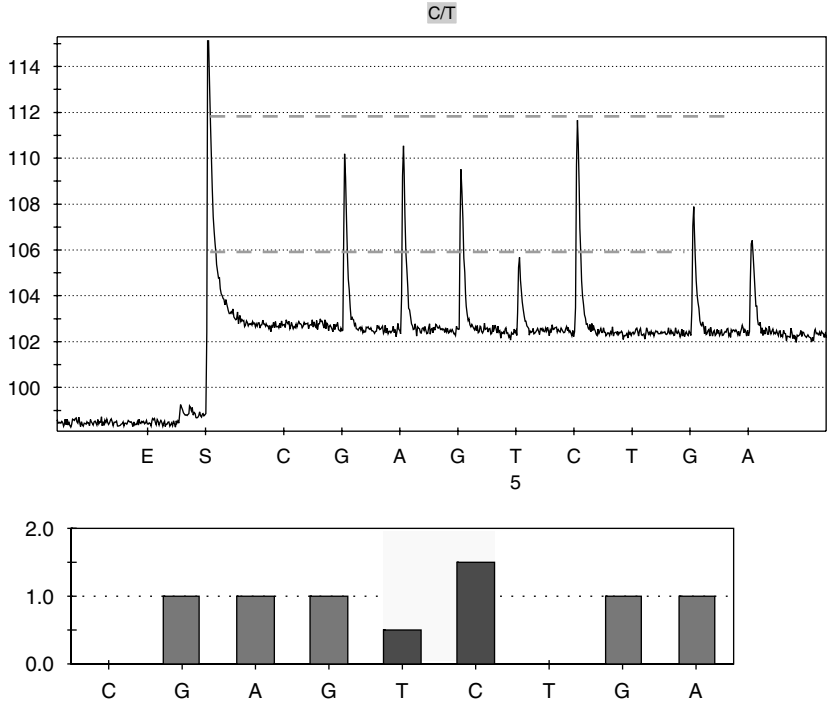

Figure 8 Example of pyrogram (up) and pattern (down) for MTHFR 677 C>T heterozygote. MTHFR, methylene tetrahydrofolate reductase.

Statistical analysis

Statistics were performed using SPSS software (Chicago, IL, USA). χ^2 test and Fisher's exact test were used for comparison of different frequencies. The α-error risk was classically chosen as 5%. Kaplan–Meier estimates and log-rank test were employed in univariate analysis of OS and PFS.

The Cox-regression method was used for toxicity risk multivariate analysis. The same method was used for response to FU-based treatment or OS and PFS multivariate analysis.

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Duality of interest

The authors declare that they have no duality of interest.

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