

Polymorphisms in the thymidylate synthase and dihydropyrimidine dehydrogenase genes predict response and toxicity to capecitabine-raltitrexed in colorectal cancer

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Abstract. Pharmacogenetics is an increasingly useful field where the genetic studies are becoming an important tool for predicting drug toxicity and/or efficacy. Thymidylate synthase (TS) and dihydropyrimidine dehydrogenase (DPD) gene polymorphisms could be highly informative tools in the clinical handling of colorectal cancer patients, who are following fluoropyrimidine based chemotherapy. Fifty-eight patients, with non-resectable metastatic colorectal cancer, were treated with capecitabine and raltitrexed, every three weeks. Patients were divided in a good-response group (complete and partial response) and a poor-response group (stable and progression). A genotype panel TS-DPD was evaluated. Results show that TS genotype analysis clearly differentiates patients with a worst response to a 5-fluorouracil based chemotherapy. DPD genotype was shown to be highly informative for prediction of toxicity of the treatment. These polymorphisms could represent an accurate, rapid and effective determination panel, indicative of resistance and toxicity for patients undergoing fluoropyrimidine based treatment.

Introduction

Colorectal cancer is the fourth most common malignancy globally and the second leading cause of cancer death in Western countries. Treatment with 5-fluorouracil (5-FU) and calcium leucovorin (LV) has been the standard therapy for over a decade. Capecitabine, a tumor-activated fluoropyrimidine carbamate potently inhibits the enzyme thymidylate

synthase (TS). Raltitrexed, a water-soluble folate-based agent, is also a TS inhibitor. Two polymorphisms have been described in the TS gene, a 28-bp double or triple tandem repeat on the enhancer region of the gene promoter (TSER), and a 6-bp deletion at the 3'UTR region (1-4). On the other hand, 70-80% of the administered 5-FU is normally degraded *in vivo* by the enzyme dihydropyrimidine dehydrogenase (DPD) (Fig. 1). A point mutation in the invariant GT splice donor site flanking exon 14, (IVS14+1G→A), causes exon skipping and leads to an inactive DPD allele (5,6). The aim of this study is to analyze TS and DPD polymorphisms and their association to a good/poor response and toxicity to 5-FU based chemotherapy.

Materials and methods

Patients. Fifty-eight patients with histological or cytological diagnosis of metastatic colorectal cancer were recruited for the study. At least one bidimensionally measurable indicator lesion, that had not been irradiated, was required. The patients were 18 years of age, or older, with a life expectancy of at least 3 months and each gave a written consent. Patients with CNS involvement of their disease, neurological or psychiatric disorders that could interfere with treatment compliance, significant cardiac disease or myocardial infarction within the previous 12 months, serious uncontrolled infections, malabsorption syndrome or patients lacking physical integrity of their upper gastrointestinal tract were excluded. Patients were also not enrolled if significant abnormalities in neutrophils ($<1.5 \times 10^9/l$), platelets ($<100 \times 10^9/l$), serum creatinine or serum bilirubin ($>1.5 \times$ upper normal limit), ALT, AST, alkaline phosphatase ($>2.5 \times$ upper normal limit) were detected. The study was conducted in concordance with the declaration of Helsinki and all current amendments. Capecitabine (1000 mg/m²) was administered (total dose of 2000 mg/m²/d) during 14 days in 3-week cycles at approximately 12-h intervals and within 30 min of a meal. Raltitrexed was administered intravenously (3 mg/m²) in 15 min in the first day and every 3 weeks. Treatment was continued until the scheduled assessment at 8 months or until the development of progressive disease if recorded earlier. Treatment interruption,

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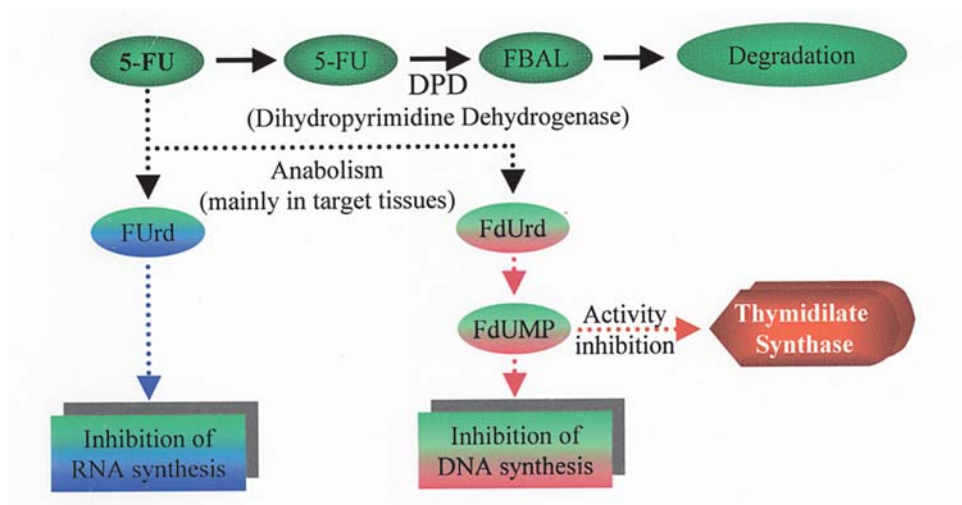


Figure 1. Pathways of 5-FU metabolism. FBAL, 5-fluoro- β -alanine; FUrđ, fluorouridine; FdUrđ, fluorodeoxyuridine; FdUMP, fluorodeoxyuridine monophosphate.

or dose reduction, was not indicated for reactions unlikely to become serious or life-threatening and following the NCIC-CTC guidelines. Tumor response was assessed according to WHO criteria (7) and confirmed at least 4 weeks later by the same evaluation.

Genotyping. DNA was extracted from peripheral blood samples (MagNA Pure LC, Roche). TS polymorphisms were analyzed as described previously (8). Primers: TSER (forward) 5'-GTGGCTCCTGCGTTTCCCC-3', TSER (reverse) 5'-GGCTCCGAGCCGGCCACAGGCATGGCGCGG-3', 3'UTR (forward) 5'-CAAATCTGAGGGAGCTGAGT-3' and 3'UTR (reverse) 5'-CAGATAAGTGGCAGTACAGA-3'. For the 3'UTR polymorphism, DraI was used as the restriction enzyme (8). Mutant DPD allele was detected by direct sequencing (9). Primers: DPD (forward) 5'-TCCTCTGCAAATGTGAGAAGGGACC-3' and DPD (reverse) 5'-TC

ACCAACTTATGCCAATTCTC-3'. A Genetic Analyzer ABI PRISM® 377 (Applied Biosystems) was used.

Statistical analysis. TSER and 3'UTR polymorphisms were first analyzed separately. The χ^2 test was used to compare the observed genotype distributions with those expected by the Hardy-Weinberg equilibrium. Secondary, χ^2 or Fischer's two-tailed exact test was used to determine the relationship between response to chemotherapy and TSER and 3'UTR genotypes of the TS gene. Finally, linkage disequilibrium between TSER and 3'UTR polymorphisms was also evaluated.

Results

Polymorphism at the TS enhancer region. Double or triple tandem repeats (28-bp) were named 2R (215 bp) and 3R (243 bp), respectively (Fig. 2A). A four tandem repeat (4R)

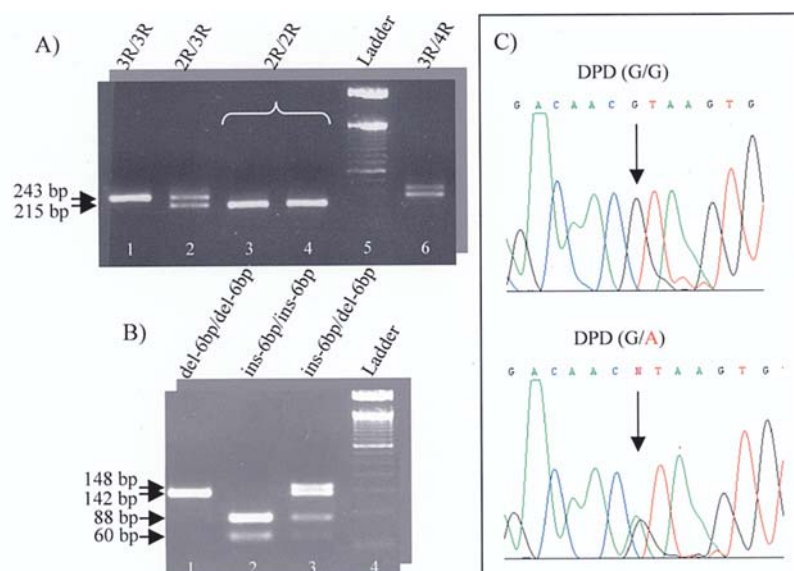


Figure 2. TS-DPD polymorphisms. (A), TSER polymorphisms (lane 5, 50 bp ladder); (B), 3'-UTR polymorphisms (lane 4, 50 bp ladder); (C), Direct sequencing of the DPD wild-type (G/G) and DPD mutant allele (G/A).

SPANDIDOS Distribution of TS genotypes within the groups studied.

	3R/3R (%)	2R/3R (%)	2R/2R (%)
Good-response patients (n=29)			
(ins-6 bp/ins-6 bp)	2	13	9
(ins-6 bp/del-6 bp)	5	20	4
(del-6 bp/del-6 bp)	-	-	-
Poor-response patients (n=26)			
(ins-6 bp/ins-6 bp)	4	4	2
(ins-6 bp/del-6 bp)	11	15	-
(del-6 bp/del-6 bp)	11	-	-

-, no patients with these genotype combinations were found.

was observed in two samples (Fig. 2A, lane 6). Due to its low frequency, those samples were excluded from further statistical analysis. The frequencies were 15% (2R/2R), 33% (3R/3R) and 52% (2R/3R), in agreement with that predicted by the Hardy-Weinberg equilibrium, and in concordance with previous studies (8,10-13).

Polymorphism at the TS 3'UTR. The genotype del-6 bp/del-6 bp shows a single fragment of 142 bp whereas the ins-6 bp/ins-6 bp genotype is characterized by two fragments of 88 and 60 bp (Fig. 2B). The 148-bp band in the heterozygous sample is caused by incomplete digestion of the ins-6 bp allele by the restriction enzyme. The distribution of the genotypes was 34% (ins-6 bp/ins-6 bp), 55% (ins-6 bp/del-6 bp) and 11% (del-6 bp/del-6 bp), which is in agreement with that predicted by the Hardy-Weinberg equilibrium. Previous studies obtained similar results (3,8,13).

DPD sequencing. Only one heterozygous subject for the IVS14+1G→A mutation was detected (Fig. 2C). The patient showed 5-FU high toxicity after the first cycle of treatment. Immediately, the 5-FU based chemotherapy was substituted by another more suitable treatment and the patient excluded from the study. The frequency of the heterozygote (1.7%) was similar to previous studies (6).

Distribution of TS polymorphisms according to the overall objective tumor response. Patients were divided in a good-response group (complete and partial response) and in a poor-response group (stable and progression). Frequencies of the TS polymorphisms are shown in Table I. The 3R/3R genotype is associated with the poor-response patients (26%), being only 7% in the good response group; 2R/2R genotype is increased (13%) in the patients showing a good response to the treatment. The statistical analysis indicated that the 3R/3R genotype was preferentially found within the poor-response group of patients ($p<0.01$) while the 2R/2R genotype was more abundant in the good-response group. On the other hand, the ins-6bp/ins-6bp genotype was frequently present in

the good-response group (24%), while the double deletion (del-6 bp/del-6 bp) was only present in the patients with a poor-response ($p<0.05$). It has been reported that TSER and TS-3'UTR polymorphisms are in linkage disequilibrium (8). We have found that among individuals with 3R/3R genotype, there are differences of statistical significance ($p<0.05$), being the genotype del-6 bp/del-6 bp segregated with the genotype 3R/3R.

Discussion

The aim of our study was to combine TS-DPD polymorphisms, that could predict response and toxicity to 5-FU based chemotherapy. Our results show that the 3R/3R genotype is clearly associated with a poor response to the treatment. It has been reported that 3R/3R is associated with a higher expression of mRNA than 2R/2R (14,15). The principle that greater levels of TS translation could protect cells from the cytotoxic effect of 5-FU has been proposed as one mechanism for tumor resistance to this drug. But there is still controversy, since studies in colon cancer cell lines have found that the TSER genotype was related to the enzyme activity, but not to protein and mRNA levels (15). Additional studies are needed to identify the regulatory factors by which this polymorphism alters the TS gene expression and/or enzyme activity. We have detected two patients with the genotype 3R/4R. Although alleles containing 4, 5 and 9 copies of the tandem repeat have also been described previously, their effect remain unclear and its frequency low in the population (10). Regarding the TS-3'UTR polymorphism, the genotype del-6 bp/del-6 bp was only present in patients showing a poor response to the treatment. It would be possible that the deletion of 6 bp in the 3'UTR region affects mRNA stability or secondary RNA structure, and could thus ultimately affect protein levels and/or regulation (3). In the context of the CRC, we have found that carrying 3R/3R and del-6 bp/del-6 bp genotypes likely predicts a poor response to 5-FU based chemotherapy, being informative and helpful in individualizing patient treatment. Clearly, other genes could be implicated in the 5-FU resistance in CRC, since 30% of the patients with a poor response to the treatment do not carry 3R/3R and/or del-6 bp/del-6 bp polymorphisms. Alterations of the cell through carcinogenesis could lead to various processes that would modify the response to the treatment. It has been published that TS polymorphisms are identical in normal and tumor tissues of homozygous individuals. However, in heterozygous samples, an imbalance between the 2R and 3R alleles in the tumor DNA was frequently observed, suggesting loss of heterozygosity (LOH) at the TS locus (16,17). This offers useful themes for undertaking larger prospective pharmacogenetic studies in the future. Regarding DPD deficiency, it is increasingly being recognized as an important pharmacogenetic factor in the aetiology of severe 5-FU associated toxicity (6,18,19). Only one heterozygous subject for this mutation was detected, indicating a low prevalence of the mutation (1.7%) in concordance with previous studies (6). Due to the high degree of 5-FU toxicity developed, the patient had to be excluded from the study. Considering the common use of fluoropyrimidines, genetic screening would be highly recommendable for the presence of the DPD gene

mutation (IVS14+1G→A) related to toxicity, prior to 5-FU administration.

In summary, our study is consistent with a model in which the TS-DPD polymorphism panel will give valuable tumor-response and toxicity information to greatly improve the selection and management of patients with CRC under a 5-FU based chemotherapy. Because only a blood sample from the patient is required, this analysis has become an easy, rapid and accurate determination in our center.

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