Potential application of GSTT1-null genotype in predicting toxicity associated to 5-fluouracil irinotecan and leucovorin regimen in advanced stage colorectal cancer patients

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Abstract. Our aim was to evaluate the role of C-69T in GSTA1, Ile105Val in GSTP1, null allele in GSTT1 and GSTM1 in the prediction of toxicity in patients treated with 5-Fu/CPT-11/Lv regimens in metastatic CRC patients. Fifty-one patients with CRC metastatic disease were analysed. All patients had bidimensionally measurable disease according to WHO criteria. The gender distribution was 37 (74%) males and 13 (26%) females; age ranged from 41 to 71 years; performance status was in all patients ≥80 (Karnofsky index). The analysis of gene polymorphism was performed in lymphocytes by using PCR-RFLP (GSTA1, GSTP1), PCR (GSTT1, GSTM1) and sequencing analysis (UGT1A1*28). An appreciable significant association was observed between the GSTT1-null and toxicity: 57% developed gastrointestinal toxicity grade III versus 23% of patients with GSTT1-present genotype (p=0.053). The other polymorphisms analysed did not show any significant relation with toxicity. Our data suggest that GSTT1-null is associated with a greater probability of developing toxicity to 5-Fu/CPT-11/Lv treatments, indicating a potential application of this genetic analysis in predicting adverse effects of this regimen.

Introduction

5-Fluouracil (5-FU) has been the mainstay of treatment for patients with advanced colorectal cancer (CRC) for more than five decades. Irinotecan (CPT-11) has been found to

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demonstrate at least equivalent efficacy to 5-FU in first-line therapy, favorable quality-of-life assessments and prolonged median survival (1,2). In Europe as well as in the USA, the combination of 5-FU plus CPT-11 is currently recommended as first-line therapy for metastatic CRC treatment (3,4). Risk factors with predictive value for toxicity have been identified in several studies. In this sense, age, performance status, bilirubinaemia, the genetic polymorphism of UDP-glucuronyltransferase-1A1 (UGT1A1) and the drug administration schedule have been show to be related with CPT-11 toxicity (5). Inter-individual differences in the pharmacokinetics of its active metabolite, SN-38, cause the variations in the effect of the drug (6). Several studies in relation with different doses and schedules of CPT-11 alone or in combination with other agents are ongoing to investigate its use as first or second-line therapy (7,8), with a view to optimising the therapeutic outcome for these patients.

On the other hand, Glutathione S-transferases (GSTs) are considered an important family of detoxifying enzymes for mutagens. They protect cellular macromolecules from damage by catalysing the conjugation of toxic and carcinogenic electrophilic molecules with glutathione. The resulting complex is less toxic and more readily excreted.

The implication of GSTs in the detoxification of heterocyclic amines (HCAs) and polycyclic aromatic hydrocarbons (PAHs) and other carcinogens has been related to GST gene alterations with colorectal cancer risk (9,10). Diverse common single nucleotide polymorphisms (SNPs) have been reported for GSTT1, GSTM1, GSTA1 and GSTP1 genes that either abolish, increase or decrease these enzyme activities (11). Homozygous inherited deletions in the GSTM1 and GSTT1 gene have been related with the reduced detoxification capacity and increased genotoxic susceptibility (12). Differential GSTT1 and GSTM1 expression have been determined in erythroid and lymphoid cells, respectively in cancer study (13). Moreover, these proteins have been localized also in the liver, the major site of drug metabolism, and colon tissue, although the expression of GSTM1 in the colon is lower compared to the liver. GSTP1 has a high expression level in the colon but is a minor component in the liver (14). The changed polymorphism Ile105Val, modifies

the enzyme affinity and activity for electrophilic substances. GSTA1 is expressed at higher levels in the liver and one single nucleotide change (C-69T) in the proximal promoter reduced expression and activity of this gene (10). GSTP1 is linked to oxidative damage to nucleic acid; whereas, GSTA1 and GSTM1 are associated to lipid peroxidation (15).

Inter-individual differences in cancer susceptibility may be mediated in part through polymorphic variability in the bio-activation and detoxification of carcinogens. In this context, GSTs genotype may influence individual states through its implications in detoxification of treatment agents, GST-mediated protection against oxidative damage during treatment and the differences in carcinogen damage to DNA. Several reports have related GSTs consistently as cancer suscepti-bility genes (9,16,17) and few studies report the toxicity relation of GSTs with chemotherapeutic agents in CRC.

The possibility of individualizing cancer treatment is gaining wide acceptance. In the present study we evaluated influence and possible relation between common null-alleles in both GSTT1 and GSTM1 genes and SNPs in GSTP1 (I105V), GSTA1 (C-69T) and the UGT1A1*28 variant respect to toxicity in patients with metastatic CRC treated with a 5-Fu/CPT-11 schedule.

Materials and methods

Patients. Fifty-one patients treated at the Universitary Hospital Reina Sofía, Córdoba, Spain, were included in this study. All patients had been diagnosed stage IV colorectal cancer and received 5-Fu/CPT-11/Lv regimens. All patients had bidimen-sionally measurable disease (according to WHO criteria) at the time of starting the treatment. Ascites and pleural effusions were not considered measurable. The gender distribution was, 38 (75%) males and 13 (25%) females with a median age of 63 (range 41-71 years). Ten patients (19%) developed a tumour in the right colon, 16 (31%) in the transverse and left colon and 25 (49%) in the rectum. Of the patients 83% presented multiple metastases, of which 57% in the liver, and 47% had received previous adjuvant chemotherapy. The performance status (PS) was classified according to Karnofsky Index (KI). The most frequent PS in patients enrolled in this study was 100 (73%), range 80-100 (Table I). All patients provided informed written consent before participating in our study.

Treatment schedule. Treatment was administered in the following schedule: 5-Fu 2.250 mg/m^2 48 h in continuous infusion (CI) and 180 mg/m^2 in 30 min of CPT-11, both every 14 days (Digestive Tumour Treatment regimen). The median number of cycles was 5.

Evaluation criteria. Physical examinations and blood counts were performed after each chemotherapy cycle. Hepatic and renal function tests and computed-tomography (CT) scans of measurable lesions were assessed at baseline and repeated every 3 months.

Toxicity. Treatment toxicity was assessed before each cycle using the National Cancer Institute Common Toxicity Criteria (NCI-CTC). We defined 'severe toxicity' as haematological or gastrointestinal toxicity of grade III-IV. If neutrophils

Table I. Patient characteristics.

Patients	Total
Sex	
Male	38
Female	13
Age (years)	
Median	63
Range	41-77
Karnofsky performance status (%)	
100	37
90	12
80	2
Primary tumour location	
Right colon	10
Transverse and left colon	16
Rectum	25
Previous adjuvant chemotherapy	
Yes	24
No	27
Metastasis	
Single	8
Multiple	43
Liver metastasis	
Yes	29
No	21

<1.5x10^9/l, platelets <100x10^9/l were observed or if there was significant persisting non-haematological toxicity, chemo-therapy was delayed until all manifestations of toxicity had disappeared. We reduced doses for chemotherapy by 20% if severe toxic effects appeared.

Supportive care included intensive treatment with loperamide for late diarrhea. Atropine was given as needed for CPT-11-related cholinergic symptoms. An antiemetic agent was provided at the discretion of the treating physician.

Treatment discontinuation. Treatment was given until disease progression, the appearance of unacceptable toxicity or patient refusal.

Genotyping. Genomic DNA was extracted from 200 µl of whole blood using the DNA Isolation Kit I from MagNa Pure LC (Roche, Barcelona, Spain) according to the protocols and software provided by the company.

GSTA1 and GSTP1 polymorphisms: Briefly, 10 µl of the genomic DNA (300 ng) was used as a template with 200 ng of primers, forward (5'-AGAATCCAGTAGGTGGCCCC-3') and reverse (3'-TGTTAAACGCTGTCACCGTCC-5') by
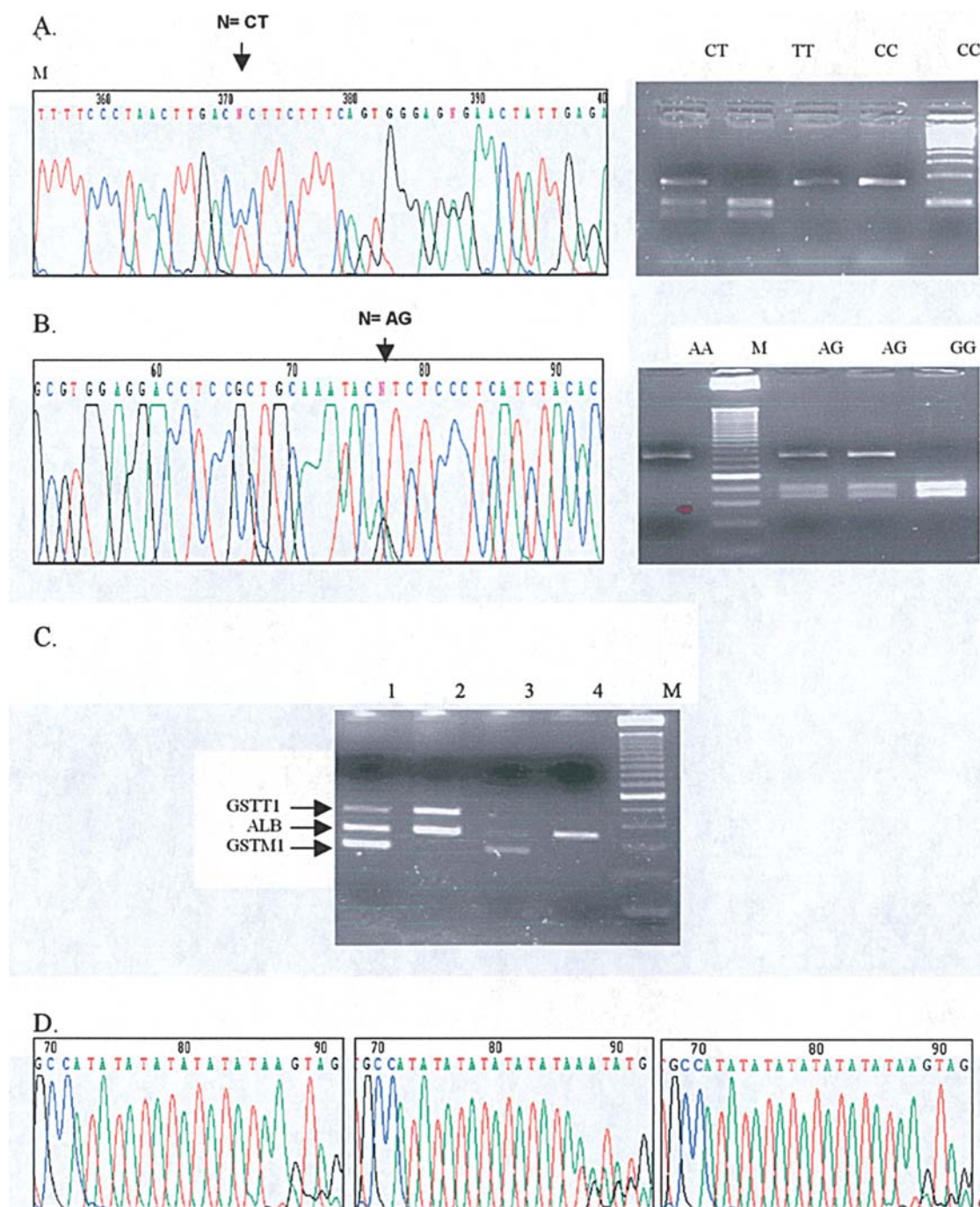
Figure 1. Digestion product. (A), GSTA1 genotype profile: heterozygote (CT), variant (TT) and wild-type (CC), and (B) GSTP1 wild-type (AA), heterozygote allele (AG) and variant (GG). (C), GSTT1 and GSTM1 allele analyses. Line 1, GSTT1 and GSTM1 present alleles. Line 2 shows the GSTT1 allele and homozygous deletion of GSTM1 and *vice versa* in line 3. Last in line 4 both GSTT1 and GSTM1 alleles are null. The molecular weight marker (M) used in (A) and (C) is of 100 bp; and 25 bp in (B). (D), Automated sequencing of the A(TA)nTAA motif, in the promoter region of the UGT1A1 gene with homozygous 6/6 (left) heterozygous 6/7 (center) and homozygous 7/7 TA repeats (right).

GSTA1 amplicon (164 bp), designed with primer express 2.0 software (Applied Biosystems, Madrid, Spain). The GSTP1 fragment polymorphism (177 bp) was analysed using primers described by Harries *et al* (18). The PCR mix containing 2.5 U AmpliTaq Gold™ (Applied Biosystems) and the rest of the PCR components in a total volume of 50 μl.

The polymorphism regions were analysed by restriction fragment length polymorphism (RFLP) technique. Digestions were carried out in a final volume of 50 μl, containing 20 μl of PCR product. Appropriate units of *Ear*I and *Bsm*AI

enzymes were added to the other components provided by New England Biolabs (NEB, Beverly, MA, USA) for GSTA1 and GSTP1 product amplified, respectively. The amplicon digestion products were analysed in an LM-Sieve 3% agarose gel (Pronadisa, Madrid, Spain) stained with ethidium bromide and visualized by UV-induced fluorescence (Fig. 1A and B).

GSTT1 and GSTM1 polymorphisms. The presence or absence (null genotype) of the GSTT1 (459 bp) or GSTM1 (273 bp)

genes was determined simultaneously using allele-specific sequence primers through a multiplex PCR protocol, according to the method of Kim *et al* (19). The absence of amplification products was consistent with the null genotypes (homozygous deletion). Control primers that amplify albumin gene (350 bp) were also included in each reaction to confirm the presence of amplifiable DNA in the samples (Fig. 1C). The resulting amplicons were separated on a 2% agarose gel (Pronadisa).

Analysis of the A(TA)nTAA motif in the promoter region of the UGT1A1 gene was performed by PCR, according to Monaghan *et al* (20), followed by automated sequencing of the purified PCR product (Fig. 1D).

Quality control. Each PCR was realized with a negative control (without DNA) to test for possible contamination. Moreover, the GSTT1 and GSTM1 study used the albumin gene (ALB) as an internal positive control. When one of these controls failed the PCR was repeated.

The GSTA1 and GSTP1 amplicon were completely sequenced to confirm the genotype observed by RFLP technique. Direct sequencing of the purified PCR products was performed on an ABI PRISM™ 377 DNA Sequencer by BigDye Terminator cycle sequencing reactions, ABI PRISM 377XL collection, DNA Sequencing Analysis 3.4.1, and Sequence Navigator software (Applied Biosystems).

Statistical analysis. Statistical analysis was performed with SPSS software 11.0 version for Windows (SPSS, Inc., Chicago, IL, USA). The χ^2 tests for contingency tables were used for statistical analysis. The level of statistical significance was set-up at $p \leq 0.05$.

The relationship between any of GSTs genotypes with toxicity and other clinical or pathological characteristics was assessed with the maximun likelihood (*LR*) test, by stepwise method.

Results

*Toxicity analysis related with GSTs, UGT1A1*28 poly-morphisms*. Individuals with GSTT1 were more frequent than GSTT1-null individuals (0.86 vs. 0.14, respectively). In contrast, a similar frequency was observed in GSTM1 and null gene (0.41 and 0.59, respectively). Four patients (8%) showed deletion of both genes.

A significantly increased proportion of GSTT1-null geno-type was detected in women (p=0.002) and GSTM1-null in men (p=0.01) (Table II). Moreover, significant association was observed with GSTT1-null genotype, 57% (4/7) digestive toxicity grade III versus 23% (10/44) of patients with GSTT1-present genotype, (p=0.053), (Table III). How-ever, after the *LR* test, the toxicity was independent of the patient gender and the relationship with the GSTT1-null was maintained.

Respect to grade III/IV toxicities, the most frequent toxi-city was diarrhoea 23.5% (12/51). Other toxicities are shown in Table IV. However, they were put together for the analysis of grade III/IV toxicities (Yes/No), joining the different haematological (anaemia, thrombocytopenia, neutropenia and febrile neutropenia) and gastrointestinal toxicities (diarrhea, mucositis and vomiting). We have to point out that 45%

Table II. Genotypes and gender frequency.

| Genotype | Gender | | P-value |
	Male	Female	
GSTT1	(Genotype %)		0.002
Present	36 (82)	8 (18)	
Null	2 (29)	5 (71)	
GSTM1			0.01
Present	12 (57)	9 (43)	
Null	26 (87)	4 (13)	
GSTA1			0.6
CC	11 (73)	4 (27)	
CT	18 (82)	4 (18)	
TT	9 (64)	5 (36)	
GSTP1			0.2
AA	15 (65)	8 (35)	
AG	20 (83)	4 (17)	
GG	3 (75)	1 (25)	
UGT1A1			0.5
6/6	15 (71)	6 (29)	
6/7	19 (73)	7 (27)	
7/7	3(100)	0	

(23/51) of the patients developed grade III toxicity (Table IV). The overall distribution of gastro-intestinal and haemato-logical toxicity was 78% (14/18) and 22% (4/18), respectively.

We have also considered other toxicities that are not related to the groups mentioned above (e.g. alopecia, nail and cardiac toxicity). Treatment with 5-FU was suspended in the case of the patient with cardiac toxicity and the symptomato-logy disappeared.

The UGT1A1*28 heterozygous and homozygous conditions were most frequent in men, but without statistical significance. No relationship existed between the UGT1A1*28 condition and gastrointestinal or haematological toxicities (Table III).

Other clinical or pathological (special number of metastasis and tumour location) characteristics were not significantly associated with these polymorphic genes (data not shown).

Discussion

Two important points must be considered with respect to therapeutic outcome for patients with advanced colorectal cancer (CRC): control of the disease and quality of life where the secondary effects of the chosen treatment are under control. The step from monotherapy to polychemotherapy has had a positive effect, reflected both in the increase of the response rate and also in survival and time to progression (3). Nonetheless, the increase in toxicity has been a limiting factor, manageable only in certain treatment schedules (21). The key to this progress seems to lie in the introduction of

Table III. Gastrointestinal (n=14) and haematological (n=4) grade III toxicity in GSTs and UGT1A1 analysed.

		AA	AG	GG	P-value
GSTP1	Gastro-intestinal	7/23 (30%)	7/24 (29%)	-	0.4
	Haematological	1/23 (4.3%)	3/24 (13%)	-	0.4
		CC	CT	TT	
GSTA1	Gastro-intestinal	4/15 (27%)	6/22 (27%)	4/14 (29%)	0.9
	Haematological	1/15 (7%)	3/22 (14%)	-	0.3
		PRESENT		NULL	
GSTT1	Gastro-intestinal	10/44 (23%)		4/7 (57%)	0.053[a]
	Haematological	4/44 (9%)		-	1
		PRESENT		NULL	
GSTM1	Gastro-intestinal	5/21 (24%)		9/30 (30%)	0.4
	Haematological	2/21 (10%)		2/30 (7%)	0.5
		6/6	6/7	7/7	
UGT1A1*28	Gastro-intestinal	6/21 (29%)	8/26 (31%)	-	0.4
	Haematological	2/21 (10%)	2/26 (8%)	-	0.8

[a]P-value ≤0.05.

Table IV. Overall treatment-related toxicity, graded according to the NCI-CTC (n=51).

Toxicity	Grade					Total grade III-IV (%)
	0	I	II	III	IV	
Neutropenia	34	5	10	2	0	2 (3.9)
Vomiting	23	13	13	2	0	2 (3.9)
Diarrheoa	17	8	14	12	0	12 (23.5)
Mucositis	35	8	6	2	0	2 (3.9)
Constipation	39	11	1	0	0	0
Hepatic	51	0	0	0	0	0
Cardiac	49	0	1	1	0	1 (1.9)
Anemia	37	13	0	1	0	1 (1.9)
Alopecia	44	1	4	2	0	2 (3.9)
Febrile neutropenia	50	0	0	1	0	1 (1.9)
Thrombocytopenia	51	0	0	0	0	0
Anorexia	46	4	1	0	0	0
Cutaneus	48	2	1	0	0	0
Ocular	49	2	0	0	0	0
Asthenia	34	13	4	0	0	0

new drugs in combination with 5-FU, such as CPT-11 or oxaliplatin.

However, it should be noted that heterogeneity in the efficacy and toxicity of chemotherapeutic agents has been observed across the human population. Administration of the same dose of an anticancer drug to a group of patients results in a range of toxicity, from the unaffected to lethal events.

Published studies report that the toxicities due to CPT-11 are primarily neutropenia and diarrhoea (22-24). In this sense, patients with the UGT1A1*28 allele (7/7 TA repeat or more) may development this toxicity easily after CPT-11 chemotherapy. In our treatment schedule, this relation was not observed. In contrast, there is a direct GSTT1 implication in toxicity development with an appreciable significant relation

between the GSTT1-null gene and general and gastro-intestinal toxicity. Our results are consistent with some recent studies but in acute myeloid leukaemia (AML) suggesting that the lack of the GSTT1 gene implies greater toxicity in these patients (25-27). Similar observations have been made in lung cancer (28). The chemotherapy agent employed in these studies are different to ours, however, determining which drug's metabolism might be influenced by GSTT1 genotype is difficult as the substrate specificity remains unclear. Geno-toxic effects of the agent diepoxybutane in GSTT1-null condition and the environmental carcinogen benzene have been reported in *in vitro* sensitivity assay (29-31).

The patients included in this study group were selected according to type, tumoural stage and treatment schedule and the frequency determined for the GSTT1 and GSTM1-null (0.14 and 0.59, respectively) gene was similar to other studies realized in Caucasian CRC groups (32-34), as well as in other European countries (GSTT1-null 0.13 in Finnish and Swedish populations and 0.58 for GSTM1-null in Portuguese populations). Although in contrast with that indicated for Spanish populations, where the loss of both genes had slightly lower frequency (35).

In our patients, the proportion of GSTT1-null was slightly more frequent in women. The frequencies coincide with another study carried out in Caucasian control populations (35). Considering the relationship between GSTT1-null and toxicity, it is possible that this loss is caused by high toxicity in woman treated with the 5-Fu/CPT-11 schedules. In this sense, it has been observed that plasma concentrations of CPT-11 and its three metabolites (SN-38, SN-38G and APC) were in general, lower in males compared with females (36). However, the toxicity was independent of the gender and the relationship with the GSTT1-null was maintained after the *LR* test.

The toxicity observed as the result of the loss of this gene is a new factor to be considered when this treatment schedule is administered to metastatic CRC patients.

Several new schemes of adjuvant therapy exist in advanced CRC patients. The pattern of inherited enzyme variants may become an additional factor to consider in deciding on the best course of therapy. The present study should be considered a preliminary finding until it has been validated in a larger study.

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