Combination of polymorphisms within 5' and 3' untranslated regions of thymidylate synthase gene modulates survival in 5 fluorouracil-treated colorectal cancer patients

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Abstract. In the present study we explored the effect of three polymorphisms of the TS gene on overall and progression-free survival of colorectal cancer (CRC) patients subjected to 5FU chemotherapy. A 28 bp variable number of tandem repeats (VNTR), a G/C single nucleotide polymorphism (SNP), and a deletion of 6 bp at position 1494 were studied. The possible combined effect of these DNA polymorphisms on the clinical outcome of patients was also evaluated. A retrospective study was carried out on paraffin-embedded sections from 113 patients diagnosed of advanced CRC. TS genotyping methods were polymerase chain reaction (PCR) for VNTR and PCR, followed by restriction length fragment polymorphism (PCR-RFLP) for SNP and ins/del 6 bp. To study the combined effect of TS polymorphisms, four categories were defined accordingly to the level of expression attributed to SNP and ins/del 6 bp genotypes: C&allele 6-, C&6+/6+, G&allele6- and G&6+/6+. VNTR and ins/del 6 bp genotypes varied with tumour anatomical site: 2R/2R genotype was rare in left-sided tumours (7.0% vs. 26.3% of right-sided and 24.1% of rectal cancers; P<0.01), where the variant allele 6- was very frequent (69.0%). Instead, most patients with right-sided tumours were wild-type homozygous 6+/6+ (63.9%) (P<0.01). Heterozygous 6+/6- genotype was more frequent among tumours classified as C (50.0%) and D (76.5%) Dukes stages (P=0.05). None of the studied polymorphisms alone affected overall or progression-free survival (PFS). C&6+/6+ and G&6+/6+ combined genotypes were respectively associated to the best and worst PFS (P=0.03 when compared with each other), while combinations carrying the allele 6- determined an intermediate evolution that might be indicative of a variable response to chemotherapy. The rate of Dukes B stage tumours was unexpectedly high (59.1%) among patients with the unfavourable G&6+/6+ combination. In our study the combination of high TS expression genotypes G&6+/6+ identifies a group of high risk within CRC patients treated with 5FU.

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Introduction

Thymidylate synthase (TS) is the therapeutic target for 5 fluorouracil (5FU) based chemotherapy, which is widely used in a variety of tumours. This enzyme is essential for DNA biosynthesis and repair, and it is directly involved in cell proliferation. TS catalyzes the reductive methylation of deoxyuridylate (dUMP) to thymidylate (dTMP), which represents the only intracellular d*e novo* source of dTMP (1). The cytotoxic effect of 5FU and its derivatives is due to their competition with dUMP. The consequence of TS blocking is a depletion of dTMP levels resulting in thymine-less death. High TS expression levels and catalytic activity have been associated to poor prognosis and response rates (2,3). The initial explanation was that elevated TS levels overcome the inhibitory effect of chemotherapeutic drugs, but latest experimental data have shown that high expression and enzyme activity have oncogenic potential (4,5).

The gene encoding TS is located at 18p11.32. Three polymorphisms affecting TS expression have been identified. A variable number of tandem repeats (VNTR) of a 28 base

pairs (bp) sequence, and a single nucleotide polymorphism (SNP) are located within the 5' untranslated region (5'UTR). The VNTR gives rise to alleles of two (2R) and three (3R) repeats, although up to nine repetitions have been described (6,7). Compared to the variant 2R allele, the wild-type 3R has shown to enhance *in vitro* the translation of TS protein because each one of the first two repeats harbours one USF family E-box consensus element. The SNP, consists of a G→C substitution at the 12th nucleotide of the second repeat of the 3R allele that abolishes its translation enhancer effect because it eliminates the extra E-box site, thus preventing the binding of USF complexes to it (8,9). Therefore, high TS expression is due to the presence of the 3G allele, while 2R/2R, 3C/3C, and 2R/3C genotypes lead to decreased TS levels (8). A 6 bp deletion within the 3'UTR, at position 1494 has also been disclosed (10). This ins/del polymorphism acts on TS expression by affecting mRNA stability, so that the deletion allele (del 6 bp, or 6-) mRNA displays a significantly shorter half-life than that of the wild-type (ins 6 bp, or 6+) (11,12).

A number of studies have been directed to elucidate if differences in survival and/or response to 5FU-based chemotherapy of patients with cancer might have a genetic background. Data on the VNTR polymorphism are mixed (13). These inconsistent results might be explained by the G/C SNP but, although it has been proposed that patients carrying the 3G allele have poorer outcome than low expressers (14,15), the reported results are not consistent (16). Data on TS 3'UTR polymorphism and survival are also heterogeneous (17-19).

The aim of our study was to investigate whether TS VNTR, SNP G/C and ins1494del 6 bp polymorphisms, either alone or in combination, affect overall and/or progression-free survival of patients with advanced colorectal cancer (CRC) treated with 5FU.

Patients and methods

Subjects and treatment. The identification of TS gene polymorphisms was carried out in a group of 113 patients diagnosed of advanced colorectal carcinoma between 1993 and 2002 and subjected to surgical resection of their tumour. Eighty-one of them had undergone adjuvant chemotherapy treatment with 5FU. The chemotherapeutic agent was administered via bolus regimen in all cases. The median of follow-up was 59 (3-148) months.

Sample collection and genotyping. Samples consisted of formalin fixed paraffin-embedded specimens of primary tumours collected from the Archives of the Pathology Department of the University Hospital of La Princesa (UAM, Madrid, Spain). Data processing was carried out so that patients' confidentiality was preserved.

DNA extraction. Genomic DNA was extracted from paraffin-embedded tissue, as described earlier (20).

TS 5'UTR polymorphisms. VNTR and SNP polymorphisms within the 5' UTR region of TS promoter were determined respectively by specific PCR, and PCR-RFLP as described earlier (14,20). As stated in previous reports based on functional studies, subjects harbouring the 3G allele (i.e., 2R/3G, 3G/3G and 3G/3C), were grouped as G patients (5'UTR high expressors), and 2R/2R, 2R/3C, 3C/3C as C patients (5'UTR low expressors) (9,14,21).

3' UTR polymorphism. The 6 bp deletion at position 1494 of the 3' UTR region was determined by a PCR-RFLP method based on that described by Ulrich *et al* (10). Briefly, 500 ng of DNA were amplified in a final reaction volume of 50 μl containing 15 pmol of each primer, 1 U of Biotools DNA Polymerase (Biotools B&M Labs., S.A, Madrid, Spain), 1X reaction buffer, 2.5 mM $MgCl_2$, and 150 μM dNTPs. An initial denaturation step of 94˚C for 5 min was followed by 40 cycles of denaturation (94˚C for 30 sec), annealing (59˚C for 45 sec) and primer extension (72˚C for 45 sec), and a final elongation step (72˚C for 5 min). The amplified fragments (10-15 μl) were digested overnight at 37 ˚C with DraI restriction endonuclease, and the fragments were electrophoresed on 4% high resolution agarose (Conda Laboratories, Madrid, Spain). Wild-type (6+) and variant (6-) alleles were ascertained respectively by the presence of two fragments of 70 and 88 bp, or one fragment of 152 bp. The heterozygous genotype displayed all of these bands, together with the 158 bp one, corresponding to residual undigested wild-type product described elsewhere (10).

PCR was carried out in a GeneAmp PCR System 2700 (Applied Biosystems, Foster City, CA, USA). All PCR primers were provided by Metabion International AG. (Martinsried, Germany). The remaining PCR reagents were supplied by Biotools B&M Labs, and restriction endonucleases were supplied by MP Biomedicals (Irvine, CA. USA).

Definition of TS5' and 3'UTR combined genotypes. Combined TS 5'UTR and 3'UTR genotypes were grouped following the classification proposed by Kawakami *et al* (21), which defines four categories basing on the level of TS expression ascribed to TS5' and 3'UTR genotypes. Namely, these groups are C&allele 6- (5'UTRLow/3'UTRLow); C&6+/6+ (5'UTRLow/3'UTRHigh); G&allele 6- (5'UTRHigh/3'UTRLow), and G&6+/6+ (5'UTRHigh/3'UTRHigh). High and low TS5'UTR expressors have been defined above; TS3'UTRLow expressers include all carriers of the variant 6- allele (i.e. homozygous 6-/6- and heterozygous 6+/6-), and TS3'UTRHigh expressors are homozygous 6+/6+ subjects.

Statistical analysis. The endpoint of the study was overall and progression-free survival, calculated from the primary surgery to the date of last follow-up or death (overall survival) or to the first evidence of disease progression (progression-free survival). Demographic and clinical variables were compared across genotype, using Fisher's exact test or the Pearson χ^2 test for categorical variables and the one-way analysis of variance for continuous and normally distributed variables or the Kruskall-Wallis test for non-normal variables. Normality was checked by the Kolmogorov-Smirnov test. To verify the agreement of the observed genotype frequencies with those expected according to the Hardy-Weinberg equilibrium model (22), the likelihood-ratio test G was used.

Table I. Baseline characteristics of the patients.

	N (%)
Age (years, median range)	66.0 (39-90)
Median OS (months)	59.0 (3-148)
Median PFS (months)	34.5 (2-147)
Gender	
Male	49 (43.4)
Female	64 (56.6)
Dukes stage	
B2	52 (46.0)
C	44 (39.0)
D	17 (15.0)
Tumour location	
Right	38 (33.6)
Transverse	3 (2.7)
Left	43 (38.1)
Rectum	29 (25.7)
Mortality	
Alive	51 (45.1)
Dead of CRC	23 (20.4)
Dead of unrelated cause	5 (4.4)
Lost	34 (30.1)

OS, overall survival; PFS, progression-free survival.

Survival (OS) and progression-free survival (PFS) curves were plotted using the Kaplan and Meier method (23) and compared with the log-rank, Breslow and Tarone-Ware tests. The estimation of overall and progression-free survival was performed only in patients treated with 5FU with an overall survival (OS) of 6 months or longer. Median follow-up time was computed for all patients alive at the time of analysis. Statistical significance was assumed for $P<0.05$ two-tail tests. All analyses were performed using the SPSS software, version 13.0 (SPSS Inc., Chicago, IL, USA).

Results

A total of 113 patients were enrolled in this study between 1993 and 2002. The median follow-up for all of them was 59 months (range 3-148). Patients characteristics are shown in Table I. Median age was 66 years (range 39-90); 43.4% were male; 71.7% received adjuvant chemotherapy.

TS polymorphisms. Genotype distributions of VNTR, SNP G/C, ins/del 6 bp polymorphisms, and their combinations are summarized in Table II. VNTR, SNP G/C and ins/del 6 bp genotyping were assessable in 113, 111 and 110 patients respectively. The frequencies of VNTR and ins/del 6 bp genotypes were in agreement with those expected accordingly to Hardy-Weinberg equilibrium model (22).

Table II. Genotype frequencies of TS polymorphisms alone and in combination.

	N (%)
VNTR (N=113)	
3R/3R	29 (25.7)
2R/3R	62 (54.9)
2R/2R	22 (19.5)
SNP (N=111)	
G	60 (53.6)
C	52 (46.4)
Ins/del 6 bp (N=110)	
6+/6+	51 (46.4)
6+/6-	52 (47.3)
6-/6-	7 (6.4)
Combined genotype (N=110)	
C&allele 6-	21 (19.1)
C&6+/6+	29 (26.4)
G&allele 6-	38 (34.5)
G&6+/6+	22 (20.0)

TS genotypes and clinicopathological variables. Table III shows the genotype frequencies of TS polymorphisms relating to Dukes stage, tumour location, number of dead and number of relapsed patients. The latter two variables refer to patients receiving adjuvant therapy with 5FU, with the exception of those dead of causes not related to cancer. The same correlations with regard to TS5' and 3'UTR combined genotypes are summarized in Table IV. No differences related to age and gender were observed (data not shown). Due to their small number, the three cases with tumours located in transverse colon are not discussed. Two of these patients were homozygous 3R/3R, and one was 2R/2R; all of them were SNP C (5'UTRLow expressors) and homozygous 6+/6+ (combined genotype C&6+/6+).

Dukes stage. The frequency of 6+/6- genotype in patients with tumours classified as C and D stages was 50% and 76.5% respectively, vs. 35.3% of B2 ($P=0.05$). No association between this variable and TS 5'UTR VNTR and SNP polymorphisms or combined genotypes was found.

Tumour location. Homozygous 2R/2R genotype was significantly less frequent in left-sided tumours (7% vs. 26.3% of right-sided and 24.1% of rectal ones; $P<0.01$). Left location was also associated to a higher proportion of 3G carriers (65% vs. 35% of C), although without reaching statistical signification ($P=0.06$). Heterozygous 6+/6- and variant homozygous genotypes were also more frequent in this site, compared to right-sided tumours (59.5% vs. 30.6% and 9.5% vs. 5.4%). In contrast, wild-type homozygous 6+/6+ was strongly associated to right-sided location (63.9%) ($P=0.04$). The link between ins/del 6 bp genotype and anatomical location of the tumour was more marked when all 6- allele

Table III. Correlation between TS polymorphisms and clinicopathological variables.

	VNTR				SNP			ins/del 6 bp			
	3R/3R	2R/3R	2R/2R	P-value	High	Low	P-value	6+/6+	6+/6-	6-/6-	P-value
Dukes stage											
B2	14 (26.9)	25 (48.1)	13 (25.0)	0.6	27 (51.9)	25 (48.1)	0.6	28 (54.9)	19 (35.3)	5 (9.8)	0.05
C	10 (22.7)	28 (63.6)	6 (13.6)		22 (51.2)	21 (48.8)		19 (45.2)	21 (50.0)	2 (4.8)	
D	5 (29.4)	9 (52.9)	3 (17.6)		11 (64.7)	6 (35.3)		4 (23.5)	13 (76.5)	0	
Tumour location											
Right	7 (18.4)	21 (55.3)	10 (26.3)		16 (43.2)	21 (56.8)		23 (63.9)	11 (36.0)	2 (5.6)	
Transverse	1 (33.3)	0	2 (66.7)	<0.01	0	3 (100)	0.06	3 (100)	0	0	0.04
Left	18 (41.9)	22 (51.2)	3 (7.0)		28 (65.1)	15 (34.9)		13 (31.0)	25 (59.5)	4 (9.5)	
Rectum	3 (10.3)	19 (65.5)	7 (24.1)		16 (55.2)	13 (44.8)		2 (41.4)	16 (55.2)	1 (3.4)	
Mortality[a]											
Dead	1 (7.7)	11 (84.6)	1 (7.7)	0.07	7 (53.8)	6 (46.2)	0.4	5 (38.5)	8 (61.5)	0	0.2
Alive	12 (29.3)	20 (48.8)	9 (22.0)		19 (46.3)	22 (53.7)		18 (43.9)	17 (41.5)	6 (14.6)	
Relapse[a]											
Yes	6 (17.6)	23 (67.6)	5 (14.7)	0.2	17 (51.5)	16 (48.5)	0.4	14 (42.4)	17 (51.5)	2 (6.1)	0.6
No	9 (30.0)	14 (46.7)	7 (23.3)		14 (46.7)	16 (53.3)		13 (43.3)	13 (43.3)	4 (13.3)	

[a]Patients treated with adjuvant chemotherapy and overall survival of 6 months or longer. Subjects dead of unrelated causes were excluded.

Table IV. Correlation between TS 5'/3'UTR combined genotype and clinicopathological variables.

	TS 5'/3'UTR combined genotype				
	C&allele 6-	C&6+/6+	G&allele 6-	G&6+/6+	P-value
Dukes stage					
B2	9 (17.6)	15 (29.4)	14 (27.5)	13 (25.5)	0.3
C	7 (16.7)	13 (31.0)	16 (38.1)	6 (14.3)	
D	5 (29.4)	1 (5.9)	8 (47.1)	3 (17.6)	
Tumour location					
Right	8 (22.2)	12 (33.3)	5 (13.9)	11 (30.6)	
Transverse	0	3 (100)	0	0	<0.01
Left	8 (19.0)	6 (14.3)	21 (50.0)	7 (16.7)	
Rectum	5 (17.2)	8 (27.6)	12 (41.4)	4 (13.8)	
Mortality[a]					
Alive	4 (30.8)	2 (15.4)	4 (30.8)	3 (23.1)	0.4
Dead	8 (19.5)	14 (34.1)	15 (36.6)	4 (9.8)	
Relapse[a]					
Yes	8 (24.2)	8 (24.2)	11 (33.3)	6 (18.2)	0.4
No	5 (17.2)	11 (37.9)	12 (37.9)	2 (6.9)	

[a]Patients treated with adjuvant chemotherapy and overall survival of 6 months or longer. Subjects dead of unrelated causes were excluded.

carriers were grouped together. These patients showed 69% of left-sided and 58.6% of rectal tumours (P<0.01). G&allele 6- combined genotype was more frequent in left colon (50%) and rectum (41.4%) than in right colon (13.9%) (P<0.01).

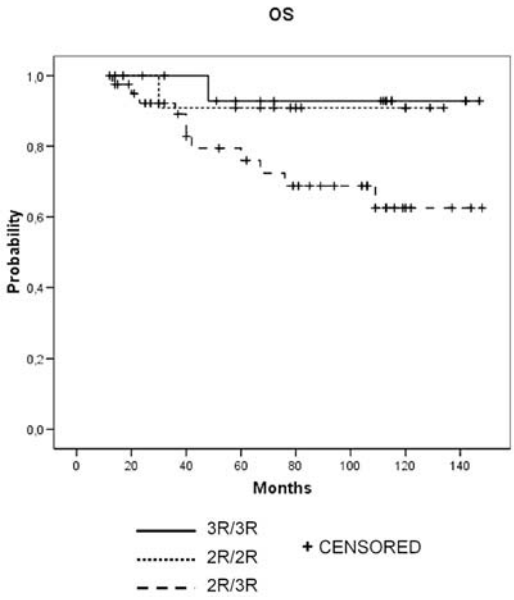

Figure 1. VNTR and overall survival (log-rank P=0.08.)

No other associations between tumour location and the remaining genotype combinations were found.

Mortality and relapsing. A trend towards higher mortality was found for 2R/3R genotype, which was present in 11/13 deceased patients (84.9%) (P=0.07). No other associations between TS genotype and number of deaths and relapses were found.

TS genotypes and survival. Median OS value was not reached in the overall group of patients included in the Kaplan-Meier analysis. Median PFS was 98 months.

TS5'UTR polymorphisms and survival. The survival analysis did not reveal any influence of VNTR genotype on PFS (log-rank P=0.4; data not shown). However, a trend to worse OS was found for 2R/3R patients (Fig. 1; P=0.08). In view of this result, the distribution of TS combined genotypes within this group was determined and compared to 3R/3R patients. The combinations carrying the 6- allele (i.e., C&allele 6- and G&-allele 6-) were present in 22/31 (71%) patients with 2R/3R genotype vs. 6/13 (46.2%) of 3R/3R, although differences were not significant (P=0.3, data not shown). SNP G/C had no effect on OS and PFS (P=0.5 and 0.4 respectively; data not shown).

TS3'UTR polymorphism and survival. Survival plots did not show any association between ins/del 6 bp genotype alone and overall or progression-free survival (P=0.3 and P=0.5; data not shown).

TS combined genotypes. Median PFS values were 36.0 months (95%CI: 0-108.5) and 22.0 months (95%CI: 6.8-37.2), respectively for patients with C&allele 6- and G&6+/6+ combined genotypes. Subjects with the remaining TS 5'/3'UTR combinations did not reach median OS or PFS values. Fig. 2 shows the survival plots. When the four groups were taken together, no association between TS combined genotype and overall or progression-free survival was observed (P=0.3 and P=0.2 respectively).

Separate analyses comparing groups by pairs were also performed (plots not shown). Log-rank P-values for OS and PFS are shown in Table V. Groups C&6+/6+ and G&6+/6+ displayed respectively the best and worst evolution. Group G&6+/6+ showed significantly shorter PFS than group C&6+/6+ (P=0.03). A similar trend was observed for OS (P=0.09). The remaining analyses did not reveal differences.

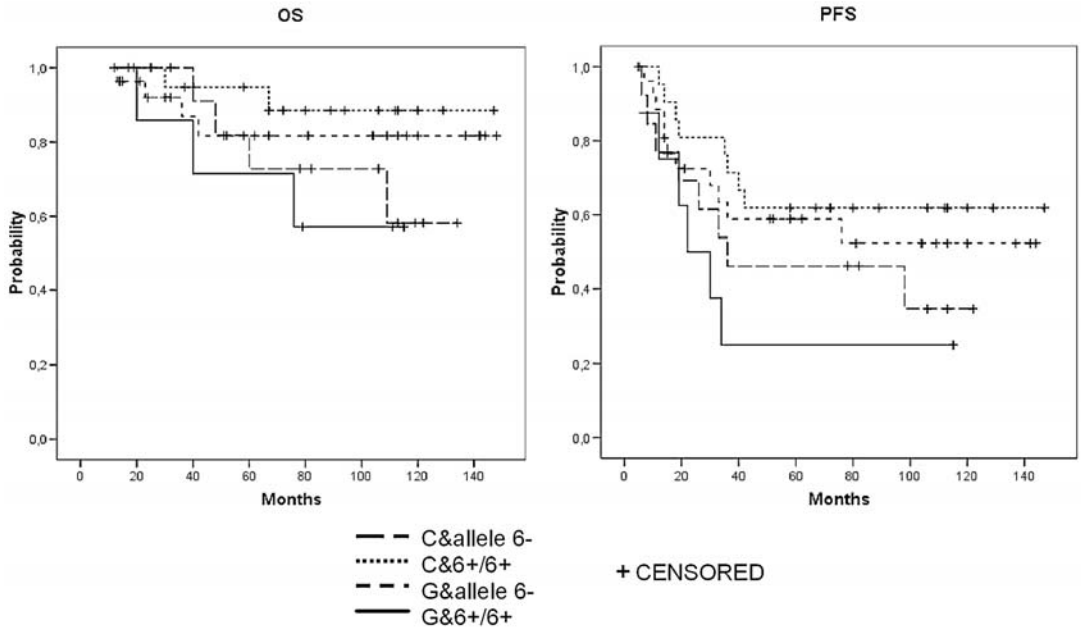

Figure 2. Overall and progression-free survival plots of TS combined genotypes. OS, overall survival (log-rank P=0.3); PFS, progression-free survival (log-rank P=0.2).

Table V. Analysis by pairs of the four TS combined genotypes.

	P-value (log-rank)	
	OS	PFS
C&allele 6- vs. C&6+/6+	0.2	0.2
C&allele 6- vs. G&allele 6-	0.4	0.4
C&allele 6- vs. G&6+/6+	0.7	0.5
C&6+/6+ vs. G&allele 6-	0.4	0.5
C&6+/6+ vs. G&6+/6+	0.09	0.03
G&allele 6- vs. G&6+/6+	0.3	0.1

Allele 6- and survival. Kaplan-Meier plots revealed that patients with combined genotypes C&allele 6- and G&allele 6- seemed to integrate a group of intermediate evolution between C&6+/6+ and G&6+/6+. As the variant allele 6- showed an association with differential clinicopathological characteristics (i.e., left tumour location, and C and D stage), we based on the premise that tumours carrying the allele 6- might display a special behaviour, and classified our patients in three groups: C&6+/6+, G&6+/6+, and 6- carriers. Fig. 3 shows the survival analyses. Significant differences were not observed when considering all the three groups, but survival plots confirmed that 6- carriers represented an intermediate situation between C&6+/6+ and G&6+/6+ combinations.

Low expression genotypes and survival. Finally, we evaluated the effect of the presence of at least one genotype of low TS expression on OS and PFS, compared with the combination of TS5'/3'UTR high expression G&6+/6+. Again, this genotype showed a trend to shorter PFS (P=0.08, Fig. 4); there were no significant differences with respect to OS. The G&6+/6+ group included in the Kaplan-Meier analysis included eight patients whose OS and PFS periods are enumerated in Table VI. Six of them (75%) relapsed and their PFS period gradually increased to a maximum of 34 months; however, the PFS of the two patients that did not relapse reached 115 months. This striking variation substantially increased the overall progression-free period of this group, and accounts for the lack of statistical differences with the remaining patients.

Characteristics of patients harbouring the combined genotypes of favourable (C&6+/6+), intermediate (G or C&Allele 6-) and unfavourable (G&6+/6+) evolution. The clinicopathological characteristics of patients carrying the allele 6- and the combinations C&6+/6+ and G&6+/6+ respectively associated to intermediate, favourable, and unfavourable outcome, were compared. The data are summarized in Table VII. There were no differences with respect to age and gender (data not shown).

The rate of left-sided tumours was higher among allele 6- carriers (49.2% vs. 22% right-sided and 28.8% rectal), while right colon and rectal cancers were more frequent in patients with C&6+/6+ and G&6+/6+ combined genotypes (41.4 and 50% respectively) (P<0.01). Number of deceased and relapsed patients did not differ significantly among the three groups. Concerning the Dukes stage, the proportion of B2, C and D tumours was comparable in allele 6- carriers (39%, B2; 39% C, and 22% D); meanwhile, the rate of B2 tumours reached 51.7 and 59.1% in patients harbouring respectively the genotype combinations of best (C&6+/6+) and worst (G&6+/6+) evolution.

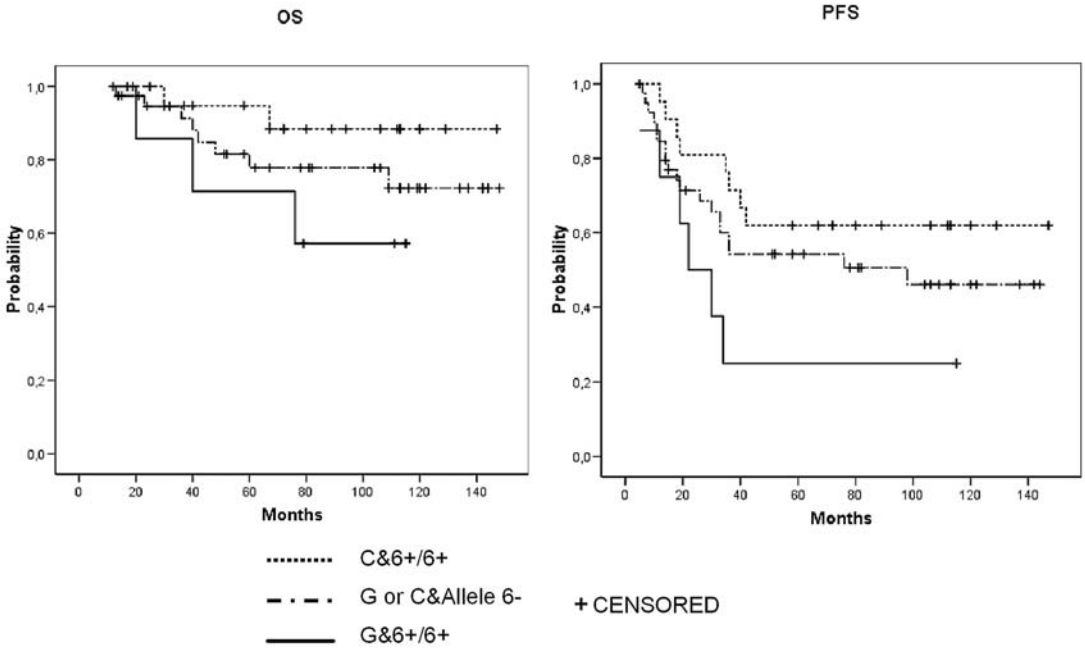

Figure 3. Overall and progression-free survival plots of combination genotypes C&6+/6+, G&6+/6+ and allele 6- carriers. Median overall survival was not reached in any of the established groups (log-rank P=0.2). Median progression-free survival was not reached by C&6+/6+ patients; it was 98 months for 6- allele carriers and 22 months (CI95%= 6.8-37.2) for group G&6+/6+ (log-rank P=0.1). OS, overall survival; PFS, progression-free survival.

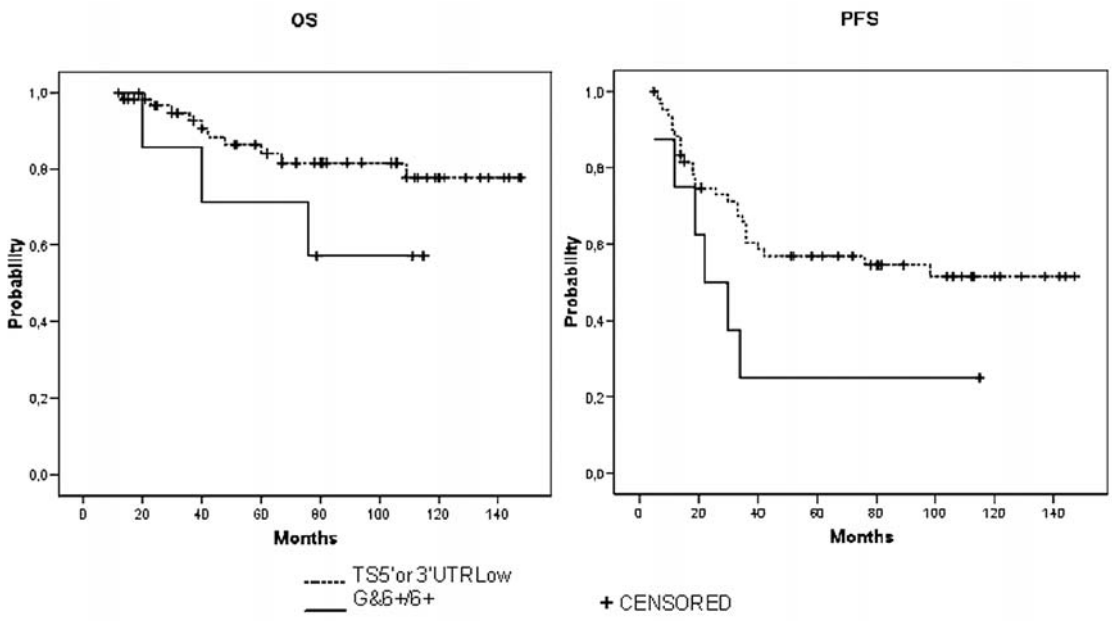

Figure 4. Overall and progression-free survival plots of carriers of at least one TS low expression genotype vs. G&6+/6+ patients. OS, Overall survival (log-rank P=0.2); PFS, progression-free survival (log-rank P=0.08)

Table VI. Values of overall and progression-free survival of TS5'/3'UTRHigh expressers (G&6+/6+) included in the Kaplan-Meier analysis.

Patient	OS (months)	Dead	PFS (months)	Relapsed
1	19	Lost	5	Yes
2	20	Yes	12	Yes
3	40	Yes	19	Yes
4	76	Yes	22	Yes
5	79	No	30	Yes
6	111	No	34	Yes
7	115	No	115	No
8	115	No	115	No

OS, overall survival; PFS, progression-free survival.

Discussion

DNA polymorphisms disclosed within TS gene have shown to influence protein expression at different levels. TS5'UTR G/C SNP acts on mRNA expression and translational efficiency (8,9,14), while TS3'UTR ins/del 6 bp involves mRNA stability (11,12). The importance of TS as therapeutic target for 5FU has favoured a number of investigations directed to elucidate the possible role of these polymorphisms as genetic markers of response to chemotherapy and/or survival (13). The variant 2R, 3C and 6- alleles have been associated to low TS expression, which has been linked to better clinical outcome of treated patients because low TS levels are expected to be better targeted by its inhibitors. In contrast, high TS levels have shown to induce transformation and tumour progression, which strongly supports an oncogenic role of this enzyme (4,5). However, in the clinical practice, the relationship between TS genotype and survival and/or response to 5FU based chemotherapy remains unclear (16).

Genotype frequencies of TS polymorphisms. In our study, the frequencies of 2R/2R, 3R/3R and 2R/3R genotypes were 19.7, 24.8 and 55.6% respectively, which is consistent with those published in Caucasians (7,25,26). VNTR is subjected to race variability: the reported rates of 2R/2R patients are around 20% in White, Black or South-Western Asian populations (3,15,18,27,28), but rarely reaches 10% in Japan, China and Korea (24,29,30), were the 3R allele is predominant (7). The same is true for the proportion of TS5'UTR high and low expressors defined by the SNP G/C accordingly to Kawakami and Watanabe (14). Such grouping is based on the effect of the G/C substitution within the second repeat of the 3R allele, that is the disruption of an extra E-box site. As a result, the levels of expression of 3C/3C and 2R/2R genotypes are comparable, while the presence of the wild-type 3G allele promotes high TS expression (14). The classification of patients as high an low expressors (in our study G and C) basing on the SNP G/C has greatly simplified the correlation of this polymorphism with clinical parameters (9,14,17). TS5'UTR high expressors represent >60% of Chinese and Japanese (9,14) patients, while its presence in Caucasians is about 50% (3,15,17,27,28). The percentages of G and C patients found by us were 52.8 and 47.2%, which is close to those reported in France (17), but slightly diverge from other studies carried out in our country, were C genotype was the most frequent (15,19).

Ethnic variations have also been reported for the frequencies of ins/del 6 bp genotypes. The proportion of 6+/6+ wild-type homozygous patients is 40-50% among

Table VII. Clinicopathological characteristics of patients with C&6+/6+, G or C&Allele 6- and G&6+/6+ combinations.

	C&6+/6+ N=29	G or C&Allele 6- N=59	G&6+/6+ N=22	P-value
Dukes stage				
B2	15/29 (51.7)	23/59 (39.0)	13/22 (59.1)	0.1
C	13/29 (44.8)	23/59 (39.0)	6/22 (27.3)	
D	1/29 (3.4)	13/59 (22.0)	3/22 (13.6)	
Tumour location				
Right	12/29 (41.4)	13/59 (22.0)	11/22 (50.0)	<0.01
Transverse	3/29 (10.3)	0	0	
Left	6/29 (20.7)	29/59 (49.2)	7/22 (31.8)	
Rectum	8/29 (27.6)	17/59 (28.8)	4/22 (18.2)	
Mortality[a]				
Yes	2/16 (12.5)	8/31 (25.8)	3/7 (42.9)	0.3
No	14/16 (87.5)	23/31 (74.2)	4/7 (57.1)	
Relapse[a]				
Yes	8/19 (42.1)	19/36 (52.8)	6/8 (75.0)	0.3
No	11/19 (57.9)	17/36 (47.2)	2/8 (25.0)	

[a]Patients treated with adjuvant chemotherapy and overall survival of 6 months or longer. Subjects dead of unrelated causes were excluded.

Caucasians, were 6-/6- are rare (around 10%). These frequencies are inverted in Asian CCR patients (26,31-33). These variations are a consequence of an imperfect linkage disequilibrium reported for 3G and 6- alleles, which are the most common among Asian subjects (21).

Genotype frequencies of TS combined genotypes. The frequencies of patients with C&allele 6-, C&6+/6+, G&allele 6-, and G&6+/6+ genotype combinations defined as a function of TS expression by Kawakami *et al* (21) were 19.1, 26.4, 34.5 and 20.0% respectively (these groups are termed as A, B, C and D in the referred study). Our figures are close to those reported by these authors in patients with gastric carcinoma (28% C&allele 6-, 21% C&6+/6+, 40% G&allele 6-, and 11% G&6+/6+), except for the slight inversion between the frequencies of groups C&allele6- and C&6+/6+, and our higher rate of patients with G&6+/6+ genotype. To our knowledge, the only study considering combinations of TS5' and 3'UTR high and low expressor genotypes in CCR patients, where TS5'UTR expression is based on the SNP G/C is that recently reported by Lurje *et al* (33). However, their data are not completely comparable to ours because heterozygous 3G carriers (2R/3G and 3G/3C), widely stated as TS5'UTR high expressors (14-16,34) are differentiated here as 'intermediate' expressors. In this investigation, only 13 out of 178 patients (7.3%) harboured the G&6+/6+ combined genotype (TS5'UTRHigh or Intermediate/TS3'UTRHigh), which is significantly less than our 20.0% (P<0.01). The inclusion in Lurje's series of 25 Asian patients, 22 out of whom (88%) were carriers of the 6- allele (i.e. TS3'UTRLow), consequently reduces the frequency of G&6+/6+ genotype and probably explains these differences.

TS polymorphisms and clinicopathological variables. Dukes stage. Ins/del 6 bp was the only polymorphism showing an association with tumour stage, heterozygous 6+/6- being more frequent in advanced stages (50% C and 76.5% D). A trend between allele 6- and advanced stage, borderline to statistical signification (P=0.055) has been observed in our country by Dotor *et al* (19). Conversely, Curtin *et al* have reported a link between the presence of variant 2R and/or 6- alleles to reduced risk of having an advanced stage tumour (31).

Tumour location. We detected strong associations between TS genotype and anatomical site of the tumour. Differences were more obvious between left and right locations. Left-sided carcinomas were characterized by a remarkably low percentage (7%) of two repeats homozygous (2R/2R), compared to right-sided (26.3%) and rectal ones (24.1%), and a high proportion of 3R/3R (41.9 vs. 18.4% right colon and 10.3% rectum). Also, carriers of allele 6- and G patients represented respectively 69 and 65.1% of cases in this site, probably reflecting the linkage disequilibrium between 6- and 3G alleles. In contrast, right-sided tumours were linked to TS3'UTR wild-type homozygous 6+/6+genotype (69%), but were not related to TS5'UTR SNP or VNTR polymorphisms. Regarding rectal tumours, the genotype distribution associated to ins/del 6 bp and SNP G/C polymorphisms, tended to follow the pattern observed for left colon, although differences were not so striking (the percentages of alleles 6- and 3G carriers were 58.1 and 55.2% respectively). As a consequence, G&allele 6- combined genotype was the more frequent in left colon and rectum (50 and 41.4% respectively, vs. 13.9% of right colon). No remarkable differences were

seen for VNTR, except for the slightly elevated rate of 2R/3R. One may speculate that these variations of genotype distribution with regard to the anatomical location might indicate that colorrectal carcinoma comprises tumours with differential characteristics in terms of origin or carcinogenic pathways. In Spain, Dotor *et al* (19) found no link between these two variables, but their results are not com-parable to ours because they grouped together left-sided and rectal tumours. A poorer prognosis has been reported for rectal carcinomas (35,36). In contrast, while some authors do not establish clear differences in the behaviour of left and right-sided tumours (32) a favourable prognosis for right colon carcinomas has been reported by others (36). Even so, the only objective fact is that, by now, we do not know the biological meaning of this association between TS genotype and tumour location.

TS polymorphisms and survival. TS 5' UTR polymorphisms. VNTR. Table III shows that 11/13 (84.6%) deceased patients treated with 5FU, with an OS of at least six months were heterozygous 2R/3R (P=0.07). The Kaplan-Meier analysis also revealed a trend to shorter OS for these patients (P=0.08), while 3R/3R and 2R/2R displayed similar behaviour. The reported data on the prognostic or predictive value of VNTR are imprecise and conflicting (8,13,18,37,38). Although better survival and/or longer time to progression have been widely linked to 2R/2R genotype, variable outcomes have been reported for 3R/3R and 2R/3R patients. Heterozygous 2R/3R genotype has been proposed as a favourable marker in some studies (30,38). In contrast, other authors have found a strong association with elevated enzyme activity, which is a proven adverse marker (3,39-41), 3R/3R predicting better response to chemotherapy (3). Combination TS 5' and 3'UTR genotypes were not taken into account in those studies. When we determined their frequencies among heterozygous 2R/3R patients, and compared them to those of homozygous 3R/3R, we found that combinations carrying the 6- allele (i.e., C&allele 6- and G&allele 6-) were present in 71% of 2R/3R patients, vs. 46.2% of 3R/3R. This might be important if we consider that, in our study, the presence of the 6- allele was linked to advanced tumour stage. Other factors that should be taken into consideration in further investigations are the variations of TS gene copy number due to loss of heterozygosis (LOH) or gene amplification. By targeted disruption of one allele, Brody *et al* (42) have shown that 3R-loss genotype leads to reduced TS protein expression and increased sensitivity to 5FU. LOH is a frequent event in carcinogenesis, and an elevated proportion of 3R-loss among presumed 3R/3R patients might explain the unexpected favourable outcome and the decreased enzyme activity reported in some studies for this genotype (3), and the poorer prognosis of 2R/3R (3,43). On the other hand, TS gene amplification has shown to increase TS expression and resistance to 5FU (44,45). Variations in TS copy number due to these mechanisms are undetectable by direct PCR methods, extensively used to screen TS genotype.

SNP G/C. In a previous investigation, we reported a link between SNP G and shorter PFS, that was borderline to statistical signification (log-rank P=0.06; Breslow P=0.04, and Tarone Ware P=0.05) (20). However, when follow-up was increased and clinical data were updated for the present study, such association was not confirmed, and this polymorphism alone did not affect either overall or progression-free survival. Despite the clear *in vitro* enhancer effect of 3G allele on TS expression, our data add more variability to the reported results about the prognostic and predictive value of this marker in the clinical practice (16,34).

TS 3' UTR polymorphisms. The Kaplan-Meier analysis did not reveal any effect of TS3' UTR genotype alone on survival, which is consistent with some studies (17,31). Instead, an association of the variant 6- allele with a reduced risk of death, together with an adverse prognosis for 6+/6+ genotype has been reported by others (19).

TS combined genotypes. Although without statistical signification, the survival analysis of the four combined genotypes pointed to a poorer outcome for patients harbouring the TS5'/3'UTR high expression combination G&6+/6+. Separate analyses comparing groups by pairs revealed marked differences in terms of PFS between C&6+/6+ and G&6+/6+ groups that were linked respectively to the best and worst prognosis. A similar trend was found regarding OS, although log-rank P-value did not reached the threshold of statistical significance. When we evaluated the effect of at least one genotype of low TS expression, G&6+/6+ patients also tended to display shorter PFS. In our study, this group was integrated by 8 patients, 6 of whom had relapsed with a PFS range of 5-34 months. However, the PFS period of the two remaining patients that did not relapse was 115 months. This dramatic difference and the small size of this group account for the lack of statistical significance in the survival analyses, and we believe that our findings support that G&6+/6+ combined genotype is a marker of poor prognosis in CCR patients treated with 5FU. Kawakami *et al*, who defined this classification for the first time, also reported that this combination, that was present in 10 subjects in their study, was associated to the poorest prognosis in gastric cancer (21). We failed to find other studies following this classification based on SNP and ins/del polymorphism. Recently, Lurje *et al* (33) have proposed another sorting were 2R/3G, and 3C/3G patients, included as high TS expressors in most studies, are considered as 'intermediate' expressors, giving rise to two additional categories. Their investigation was carried out in stages II and III CCR patients subjected to adjuvant chemotherapy with 5FU. The differentiation of TS5'UTR intermediate expressors makes unsuitable to compare their data of survival with ours. Moreover G&allele 6- and G&6+/6+ combined genotypes (in their study 'TS5'UTR high/TS3'UTR high or low') were grouped together for the Kaplan-Meier analysis. Even so, the authors also concluded that the 3G/6+ haplotype is a marker of poor prognosis and is associated to the greatest risk of recurrence.

In contrast to us, both Kawakami (21) and Lurje (33) observed that the best prognosis corresponded to TS5'/3'UTRLow expressor (group C&allele 6-) patients with gastric cancer and colorectal carcinoma. In our study, combination genotypes harbouring the 6- allele seemed to be

associated to an intermediate evolution. Based on the survival plots and on the previous observations that tumours carrying the deletion allele displayed differential clinico-pathological features (left location, advanced stage), we regrouped the patients as C&6+/6+, G&6+/6+ and 6- carriers (G or C&allele 6-). The Kaplan-Meier test confirmed this 'intermediate' behaviour of 6- carriers, suggesting that the deletion modulates the effect of SNP on response to chemotherapy. The 'low TS expression' associated to 6- allele is due to an impaired mRNA stability. This is caused by the decay-promoting RNA binding protein (RBP) AUF-1, which displays preferential affinity for the variant 6- mRNA. A number of RBPs join to both 6- and 6+ mRNAs, but only AUF-1 binding to the variant transcript is significantly stronger than the union to the wild-type messenger. The consequence is a decrease in 6- mRNA levels that represents 4- to 6-fold that of the wild-type ones (12). It is likely that this phenomenon affects the grade of expression determined by G/C SNP. This is specially important, given that TS is the only eukaryotic protein whose expression is controlled by binding to its own mRNA (46), because messenger stability might particularly affect this unique post-translational regulatory mechanism. In our study, C&6+/6+ and G&6+/6+, or, in other words, C and G patients with 'stable' 6+ mRNA, display a substantially different behaviour in terms of PFS, being respectively associated to the best and worst prognosis, as expected if considering the data of functional studies on the SNP G/C. In contrast, the survival plots of C and G patients with 'unstable' 6- mRNA are very close and integrate a group of intermediate evolution between C&6+/6+ and G&6+/6+. This finding might be indicative of the suggested inter-ference of the variant 6- transcript on the response to 5FU based chemotherapy.

Table VII shows the characteristics of patients with combined genotypes C&6+/6+ (good prognosis), G&6+/6+ (bad prognosis) and G or C&Allele 6- (intermediate prognosis). There are no appraisable differences among them, with the exception of the higher frequency of left-sided tumours among 6- carriers, already discussed. However, surprisingly, the rate of B2 stage tumours reached 59.1% of G&6+/6+. As Dukes stage B is accepted as an independent marker of better prognosis, the proportion of B2 tumours was unexpectedly elevated in a group of patients carrying a genotypic combination that resulted associated to the worst outcome.

In summary, our data support that G&6+/6+ (TS5'/3'UTR high expression) combined genotype identifies a group of poor prognosis among advanced CCR patients treated with 5FU based adjuvant chemotherapy, while the presence of the 6- allele is linked to variable response to treatment. Moreover, if confirmed in further studies, the biological meaning of the association of certain TS genotypes with particular tumour locations should be investigated.

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