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ORIGINAL ARTICLE

Retrospective Study

Integrating *TYMS*, *KRAS* and *BRAF* testing in patients with metastatic colorectal cancer

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Abstract

AIM

To investigate the impact of thymidylate synthase (*TYMS*), *KRAS* and *BRAF* in the survival of metastatic colorectal cancer (mCRC) patients treated with chemotherapy.

METHODS

Clinical data were collected retrospectively from records of consecutive patients with mCRC treated with fluoropyrimidine-based chemotherapy from 1/2005 to 1/2007. Formalin-fixed paraffin-embedded tissues were retrieved for analysis. *TYMS* genotypes were identified with restriction fragment analysis PCR, while *KRAS* and *BRAF* mutation status was evaluated using real-time PCR assays. *TYMS* gene polymorphisms of each of the 3′ untranslated region (UTR) and 5′UTR were classified into three groups according to the probability they have for high, medium and low *TYMS* expression (and similar levels of risk) based on evidence from previous studies. Univariate and multivariate survival analyses were performed.

RESULTS

The analysis recovered 89 patients with mCRC (46.1% *de novo* metastatic disease and 53.9% relapsed). Of these, 46 patients (51.7%) had colon cancer and 43 (48.3%) rectal cancer as primary. All patients were treated with fluoropyrimidine-based chemotherapy (5FU or capecitabine) as single-agent or in combination with irinotecan or/and oxaliplatin or/and bevacizumab. With a median follow-up time of 14.8 mo (range 0-119.8), 85 patients (95.5%) experienced disease progression, and 63 deaths (70.8%) were recorded. The 3-year and 5-year OS rate was 25.4% and 7.7% while the 3-year progression-free survival rate was 7.1%. Multivariate analysis of *TYMS* polymorphisms, *KRAS* and *BRAF* with clinicopathological parameters indicated that *TYMS* 3′UTR polymorphisms are associated with risk for disease progression and death ($P < 0.05$ and $P < 0.03$ respectively). When compared to tumors without any del allele (genotypes ins/ins and ins/loss of heterozygosity (LOH) linked with high *TYMS* expression) tumors with del/del genotype (low expression group) and tumors with ins/del or del/LOH (intermediate expression group) have lower risk for disease progression (HR = 0.432, 95%CI: 0.198-0.946, $P < 0.04$ and HR = 0.513, 95%CI: 0.287-0.919, $P < 0.03$ respectively) and death (HR = 0.366, 95%CI: 0.162-0.827, $P < 0.02$ and HR = 0.559, 95%CI: 0.309-1.113, $P < 0.06$ respectively). Additionally, *KRAS* mutation was associated independently with the risk of disease progression (HR = 1.600, 95%CI: 1.011-2.531, $P < 0.05$). The addition of irinotecan in 1st line chemotherapy was associated independently with lower risk for disease progression and death (HR = 0.600, 95%CI: 0.372-0.969, $P < 0.04$ and HR = 0.352, 95%CI: 0.164-0.757, $P < 0.01$ respectively).

CONCLUSION

The *TYMS* genotypes ins/ins and ins/LOH associate with worst prognosis in mCRC patients under fluoropyrimidine-based chemotherapy. Large prospective studies are needed for validation of our findings.

Key words: Thymidylate synthase; Polymorphisms; mCRC; Loss of heterozygosity; Survival; Chemotherapy; *KRAS*; *BRAF*; *TYMS*

Core tip: The etiology of resistance to new targeted agents and chemotherapy is currently being investigated based upon the patients' genetic profile in order to develop a prognostic model that could lead to individualized treatment. In this context, we studied the effect of thymidylate synthase (*TYMS*) polymorphisms that have been described so far, taking into account the presence of *KRAS* and *BRAF* mutations in association with the treatment. *TYMS* 3′ untranslated region polymorphism ins/ins and ins/loss of heterozygosity emerged as an independent factor that increases the risk of both disease progression and death. Regimens that included irinotecan had reduced risk of disease progression and death.

Ntavatzikos A, Spathis A, Patapis P, Machairas N, Peros G, Konstantoudakis S, Leventakou D, Panayiotides IG, Karakitsos P, Koumarianou A. Integrating *TYMS*, *KRAS* and *BRAF* testing in patients with metastatic colorectal cancer. *World J Gastroenterol* 2017; 23(32): 5913-5924 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i32/5913.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i32.5913

INTRODUCTION

Metastatic colorectal cancer (mCRC) is the second and third leading cause of cancer-related death in Europe[1] and the United States[2] respectively, although important and constant overall survival (OS) improvements have been achieved[3] in the last decades. Even though today there are more treatment options, including several basic chemotherapy regimens in combination with targeted agents[4], it has been found that large variation exists in individual patient prognosis and response to chemotherapy, caused by molecular heterogeneity[5]. As a result, treatment decisions are more complex and largely

empirical[6]. This depicts our lack of understanding of the molecular background and the interplay of different oncogenic pathways, such as *RAS* and *BRAF*, with gene polymorphisms, such as thymidylate synthase (*TYMS*), that can be responsible for the heterogeneity of responses to treatments.

KRAS is a member of the *RAS* family of genes (*KRAS, NRAS* and *HRAS*) that encode quanosine-5'-triphosphate (GTP)-binding proteins, which acts as a molecular switch, linking receptor and non-receptor tyrosine kinase activation to downstream cytoplasmic or nuclear events. Activating mutations in RAS result in stimulating cell proliferation and inhibiting apoptosis. Around 32%-40% of CRC harbor a *KRAS* mutation[7,8] which is a predictor of response to anti-EGFR treatment[9,10]. *BRAF* is a *KRAS* downstream abnormally activated kinase that has been shown to have a similar adverse effect on treatment response[7,8].

The backbone of mCRC chemotherapy are fluoropyrimidines (5-FU and capecitabine) that cause inhibition of *de novo* thymidine creation from uracil by the TYMS enzyme. Potential resistance mechanisms to fluoropyrimidines include *TYMS* gene amplification[11], loss of heterozygosity (LOH)[12] and a negative feedback mechanism[13]. The *TYMS* gene (GeneID 7298[14]) is located on the short arm of chromosome 18 (18p11.32) and several polymorphisms of the *TYMS* gene have been connected to variable TYMS protein levels and therapeutic outcome in relation to 5-FU.

The first polymorphism has been identified in the 5' untranslated region (UTR) and includes an insertion of a 28 base-pair (bp) repeat (rs34743033[14]), that adds an extra binding site for the upstream stimulatory factor-1 (USF-1) transcription factor (E-box CACTTG[15]). This USF-1 extra binding site acts as an enhancer to the *TYMS* promoter which leads to increased *TYMS* expression and thus to increased TYMS enzyme activity[16]. This results in alleles with two or three 28 bp tandem repeats (2R or 3R respectively). The second polymorphism (rs2853542[14]) is a G>C single nucleotide polymorphism (SNP) in the second 28 bp repeat of 3R alleles that abolishes the extra USF-1 binding site[17] and leads to conversion of the transcriptional activity from a 3R to a 2R. The third polymorphism is located on the 3'UTR (rs34489327[14]) and is a 6 bp insertion linked to stabilization of the mRNA transcript[18,19]. The above polymorphisms produce three genotypes: ins/ins (homozygous for insertion of 6bp), del/del (homozygous for deletion of the 6bp) and ins/del (heterozygous).

This study aims to investigate the associations of *TYMS* polymorphisms, LOH, *KRAS*/*BRAF* mutations and clinicopathologic characteristics with the survival outcomes of patients with mCRC treated with 1st line fluoropyrimidine-based chemotherapy.

MATERIALS AND METHODS

Patients and clinical data

This was a retrospective study carried out by a single institution (University General Hospital "ATTIKON", Athens, Greece). Clinical data were collected from records of consecutive patients with mCRC treated with fluoropyrimidine-based chemotherapy from 1/2005 to 1/2007. Formalin-fixed paraffin-embedded tissues (FFPE) from consecutive patients with mCRC were retrieved for analysis.

The study protocol was approved by the Institutional Review Board and Ethical Committee (University General Hospital "ATTIKON").

DNA extraction

Five 5-μm thick FFPE sections from a site containing at least 30% malignant cells were used for DNA extraction by means of a commercially available kit (Purelink Genomic DNA Kit; Thermo Fisher Scientific, Schwerte, Germany). DNA was quantified using qPCR (Quant-iT™ PicoGreen® dsDNA Assay Kit; Thermo Fisher Scientific) and was diluted accordingly to achieve a concentration of 10 ng/μL for *TYMS* polymorphisms and 4 ng/μL for *KRAS* mutation detection.

TYMS polymorphisms

Analysis was performed as previously described with minor modifications[20]. PCR was performed using 1 U of Platinum® *Taq* DNA Polymerase (Thermo Fisher Scientific), 1.5 mmol/L of Mg, 200 nmol/L of dNTPs, and primers. The same primers were used, but 5' UTR amplification was performed using a GC-rich amplification kit (PCRX Enhancer System; Thermo Fisher Scientific) adding 1 × of PRCx Enhancer. Genotyping for the 2R/3R polymorphism was performed by running 10 μL of the PCR product on a 1.5% agarose gel and staining with EtBr (Figure 1). For the 12 G>C substitution, 10 μL of PCR product was digested with 1 U of *Hae*III (Takara, Shiga, Japan) for 1 h at 37 ℃ and run on an 8% 19:1 polyacrylamide gel (Figure 2).

The 3'UTR was also analyzed on polyacrylamide gels (Figure 3). LOH analysis was performed *via* analyzing the intensity of the 5'UTR and 3'UTR bands on the pictures acquired using GeneTools software (Syngene, Cambridge, United Kingdom) (Figures 1 and 3). When either of the bands had an intensity of < 50% of the other, the sample was categorized as having a LOH. Samples showing LOH were defined as 2R/3RGLOH, 2RLOH/3RG, 2R/3RCLOH and 2RLOH/3RC to indicate the allele that was partially lost. Selected products were sequenced to verify the sequence amplified. Blast of the sequenced products and alignment with the latest human assemblies revealed that the amplified product was 242 bp for 3R and 214 bp for 2R genotypes.

Mutation detection

Detection of *KRAS* mutations of codons 12 and 13 was performed with a commercially available Real-Time (RT) PCR kit (Therascreen *KRAS*; DxS Diagnostics, Manchester, United Kingdom) detecting six mutations of codon 12 (G12D, G12A, G12V, G12S, G12R, G12C)

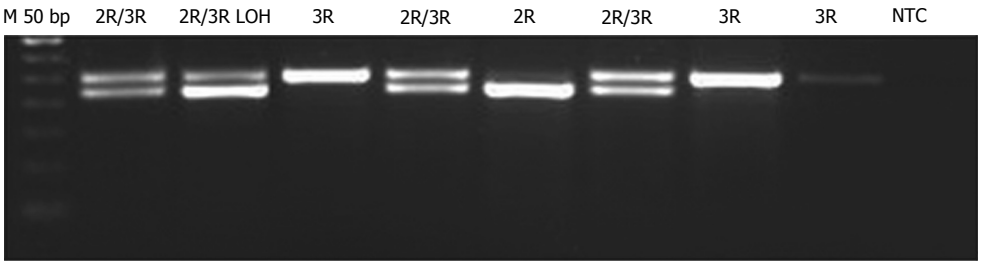

Figure 1 Agarose gel with PCR products for the thymidylate synthase 5'untranslated region 28 bp insertion. A DNA ladder of repeated 50 bp fragments was used (M 50 bp). All potential genotypes (2R 241 bp and 3R 242 bp) are depicted as well, as a sample with LOH for 3R (Lane 3). bp: Base pair; LOH: Loss of heterozygosity; UTR: Untranslated region.

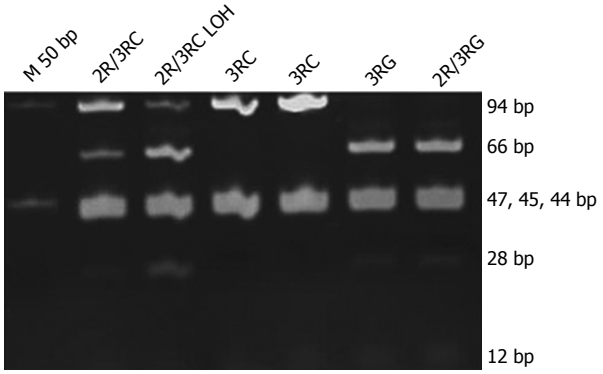

Figure 2 Polyacrylamide gel resolution showing the 12G>C substitution in the 5'untranslated region after digestion. Expected bands are 12, 44, 45 and 47 bp for all genotypes. Digestion of a sample with 2R or 3R12G genotype results in production of two bands of 66 and 28 bp, while in 3R12C genotypes those two fragments are left undigested in a single 94 bp fragment. bp: Base pair; LOH: Loss of heterozygosity; UTR: Untranslated region.

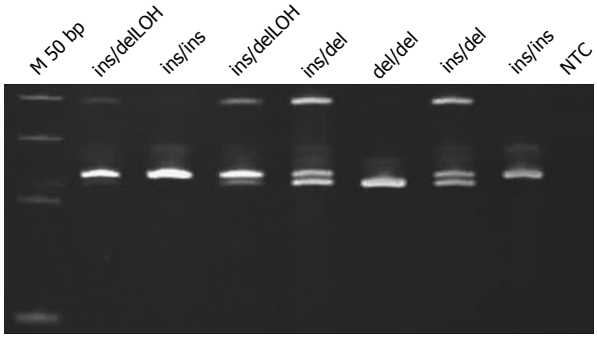

Figure 3 Polyacrylamide gel resolution showing the 3'untranslated region products. Expected bands are 104 and 110 bp. In heterozygotes, a second band of approximately 200 bp was observed due to heteroduplex mismatches. bp: Base pair; UTR: Untranslated region.

and one mutation of codon 13 (G13D)[21]. A positive reaction mix for all mutations was included. A second exogenous reaction was simultaneously taking place, to avoid false negative results caused by PCR inhibitors. Samples were characterized as bearing a mutation only if ΔCt (Ct of control reaction - Ct mutation reaction) was lower than the value set by the manufacturer.

The activating mutation V600E of *BRAF* was identified using molecular beacons, as previously described[21]. One beacon for the wild-type and one for the mutant allele were added at a final concentration of 100 nmol/L in a 25 μL PCR reaction containing 1 × PCR Buffer, 6 mmol/L $MgCl_2$, 200 nmol/L dNTPs, 300 nmol/L of each primer and 1U of Platinum® *Taq*. The PCR thermocycling profile used was 95 ℃ for 2 min, followed by 40 cycles of 95 ℃ for 10 s, 62 ℃ for 60 s and 72 ℃ for 20 s. SKMEL2 and SKMEL20 DNA extracts were used as positive controls for both the wild-type and mutant allele (CLS, Germany). All RT-PCR experiments were performed on an ABI 7500 Fast instrument (Thermo Fisher Scientific).

TYMS gene polymorphism stratification model

Polymorphisms of the *TYMS* gene in each the 3'UTR and 5'UTR were classified into three groups according to the probability they have for high, medium and low TYMS expression (and similar levels of risk[22]), taking into account the following evidence from previous studies: (1) 3R polymorphism has higher translation efficiency than that of the 2R, leading to higher TYMS protein expression associated with resistance to 5FU-based chemotherapy[18,23], while the 2R/3R has an intermediate TYMS protein expression profile[24]; (2) the SNP G>C results in the 3RC genotype, reported to display a similar transcriptional activity as the 2R genotype (since 3RC and 2R have the same number of binding sites for the USF-1)[17,25]; (3) the 6 bp insertion, located in the 3'UTR of the *TYMS* primary transcript, favors the *TYMS* mRNA stability, increasing TYMS protein expression[26] and the possibility of resistance to 5FU[18]; (4) Lower TYMS protein expression leads to higher sensitivity to fluoropyrimidine-based therapy[27,28]; and (5) LOH is associated with a higher risk of resistance to 5FU chemotherapy[12,29]. Genotypes categorized into expression groups are shown in Table 1.

Statistical analyses

OS was defined as the time from the initiation of 1st line chemotherapy to the date of death by any cause. Progression-free survival (PFS) was calculated as the time from 1st line chemotherapy initiation to the date of verified progression of the disease or the date of death by any cause. Surviving patients were censored at the date of last contact.

The relationship of *TYMS* polymorphisms groups with OS and PFS was assessed by univariate Cox regression analysis. Time-to-event distributions were

Table 1 Thymidylate synthase polymorphisms' groups per untranslated region

	Low expression	Medium expression	High expression
TYMS 3'UTR	del/del	ins/del del/LOH	ins/ins ins/LOH
TYMS 5'UTR	2RG 2RG/3RC 3RC	2RG/3RG 2RG/3RG 2RG/3RCLOH 2RG/3RGLOH 2RGLOH/3RC	3RG 3RG/3RC 2RGLOH/3RG

LOH: Loss of heterozygosity; UTR: Untranslated region.

Table 2 Clinicopathologic data for patients with metastatic colorectal cancer *n* (%)

Clinicopathologic data	Relapses	*De novo* metastatic	Total
	48 (53.9)	41 (46.1)	89 (100)
Age	65 (40-84.1)	64 (27-86)	65 (27-86)
Male	34 (70.8)	23 (56.1)	57 (64.8)
Primary site			
Colon	20 (41.7)	26 (63.4)	46 (51.7)
Rectum	28 (58.3)	15 (36.6)	43 (48.3)
Histological grade			
Ⅰ + Ⅱ	27 (56.3)	28 (68.3)	55 (61.8)
Ⅲ + Ⅳ	21 (43.7)	13 (31.7)	34 (38.2)
KRAS mutation	22 (45.8)	18 (43.9)	40 (44.9)
BRAF V600E mut	2 (4.2)	3 (7.3)	5 (5.6)
TYMS LOH	15 (31.3)	11 (26.8)	26 (29.2)
Fluoropyrimidine-based CT			
Monotherapy or with	5 (10.4)	5 (12.2)	10 (11.2)
Irinotecan	18 (37.5)	10 (24.4)	28 (31.4)
Oxaliplatin	22 (45.8)	22 (53.7)	44 (49.4)
Oxaliplatin and irinotecan	3 (6.3)	3 (7.3)	6 (6.7)
Bevacizumab	31 (64.6)	30 (73.2)	61 (68.5)
No chemotherapy	0 (0.0)	1 (2.4)	1 (1.1)
Overall survival			
Deaths	30 (62.5)	33 (80.5)	63 (70.8)
Time[1] in mo	21.4 (12.2-30.6)	18.2 (14.3-22.0)	19.8 (15.8-23.9)
Progression-free survival			
Events	44 (91.7)	41 (100.0)	85 (95.5)
Time[1] in mo	10.8 (9.0-12.5)	9.9 (7.0-12.8)	10.6 (8.8-12.5)
Follow-up in mo	14.2 (0-72.5)	17.0 (0.8-119.8)	14.8 (0-119.8)

Data are presented as *n* (%) or median (range) or [1](95%CI). CT: Chemotherapy; LOH: Loss of heterozygosity.

estimated using Kaplan-Meier curves. Correlation of *TYMS* polymorphisms among them and with selected clinicopathological characteristics were performed using the χ^2 test. For all correlations, the level of statistical significance was set at $P = 0.05$.

The Cox proportional hazards model was used to assess the relationship of clinicopathological parameters and the examined polymorphisms with OS and PFS. In the multivariate Cox regression analysis, a backward selection procedure with a removal criterion of $P > 0.10$ based on likelihood ratio test was performed to identify significant variables among the following: age, sex (female *vs* male), histological grade (Ⅲ-Ⅳ *vs* Ⅰ-Ⅱ), primary site (rectal *vs* colon), *KRAS* and *BRAF* status, groups of *TYMS* polymorphisms, existence of LOH, history of relapse or *de novo* metastatic disease and treatment.

Statistical analysis was conducted using SPSS software for Windows (version 24; SPSS Inc, Chicago, IL, United States).

RESULTS

Patient and tumor characteristics

Patients' information including age, sex, primary tumor site, histological grade, treatment and survival are presented in Table 2. The median age was 65 years (range: 27-86), and the primary site was colon or rectum in 46 and 43 patients respectively. *De novo* metastatic disease was present in 41 patients (46.1%). First-line fluoropyrimidine-based chemotherapy was administered to 88 patients, with a median number of 6 cycles (range: 1-12). In total, 5FU-based chemotherapy was given to 13 patients (14.6%), while 75 patients (84.3%) received capecitabine-based chemotherapy. Fluoropyrimidine-based regimens were combined with irinotecan (31.4%), oxaliplatin (49.4%) or both drugs (6.7%). Bevacizumab was included in the 1st line treatment of 61 patients (68.5%). With a median follow-up of 14.8 mo (range: 0-119.8), 85 patients (95.5%) experienced disease progression and 63 deaths (70.8%) were recorded. The 3-year and 5-year OS rate was 25.4% and 7.7% respectively, while the 3-year PFS rate was 7.1%.

TYMS genotypes

The detected genotypes of *TYMS* according to *de novo* metastatic or relapsed patients are shown in Supplemental Table 1. The wide variations deriving from *TYMS* polymorphism combinations and the presence of LOH according to *de novo* metastatic and relapsed patients are shown in Supplemental Table 2. The 3'UTR polymorphisms had no association with the 5'UTR polymorphism or the SNP G>C. The ins alleles correlated almost statistically significantly with LOH, as shown in Supplemental Table 3.

Analysis of significant association of *TYMS* polymorphisms with patient and tumor characteristics is shown in Table 3. Younger patients (< 65 years old) were more frequently found to carry 2R, but not in a statistically significant way. Also, low grade tumors (Ⅰ, Ⅱ) associated with 2RG/3RG ($P < 0.05$). The absence of mutations in *KRAS* correlated with 3RG/3RC ($P < 0.04$).

Correlations of clinicopathological features and genotype with survival outcomes

Analysis of patients according to *TYMS* expression groups and genotypes are shown in Table 4. Univariate Cox regression analysis of clinicopathological parameters in relation to PFS and OS showed no significant

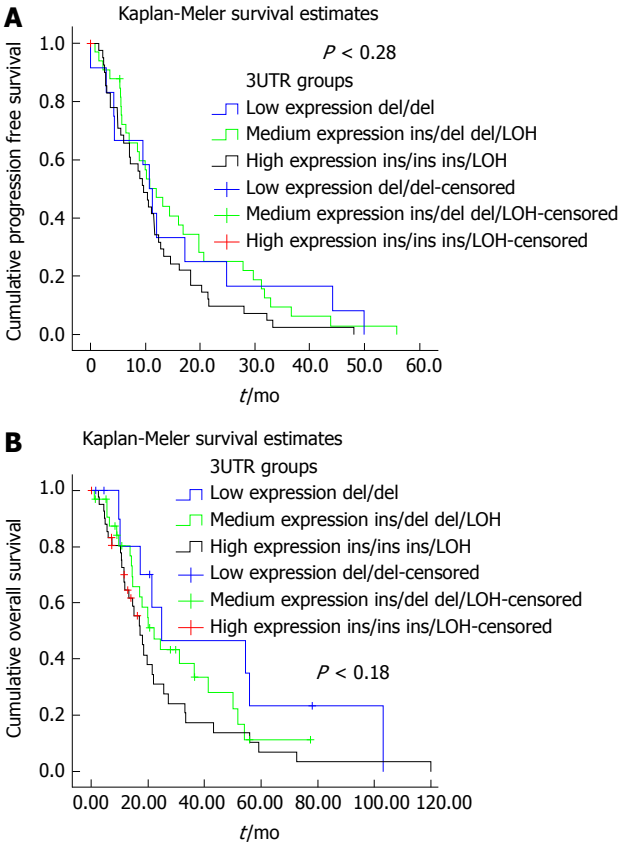

Figure 4 Kaplan-Meier survival curve. A: Kaplan-Meier curve for PFS according to *TYMS* 3'UTR groups; B: Kaplan-Meier curve for OS according to *TYMS* 3'UTR groups. Comparisons were made using long-rank tests. OS: Overall survival; PFS: Progression-free survival; UTR: Untranslated region.

association in our set of data. Univariate Cox regression analyses of *TYMS* polymorphisms and groups, *KRAS* and *BRAF* mutations and LOH are shown in Table 5. The univariate analysis of *TYMS* 3'UTR polymorphisms and LOH demonstrated a trend of lower risk for disease progression and death for the genotypes del/del, ins/del and even ins/LOH compared with ins/ins. There is a trend for increased risk of death for patients with *KRAS* mutation. The analysis of *TYMS* 5'UTR polymorphisms, whether taking into consideration the SNP G>C and LOH or not, also showed no significant effect.

Multivariate analysis of *TYMS* polymorphism groups and selected clinicopathological parameters is shown in Table 6. *KRAS* mutation, existence of LOH and the group of *TYMS* polymorphisms ins/LOH - ins/ins were associated with increased risk for disease progression, while the addition of irinotecan in the 1st line chemotherapy was associated with lower risk. In terms of OS, the group of *TYMS* polymorphisms ins/LOH - ins/ins was associated with increased statistical risk both of disease progression and death. Kaplan-Meier curves for PFS and OS according to *TYMS* 3'UTR polymorphisms groups are shown in Figure 4A and B respectively. Furthermore, the addition of irinotecan or oxaliplatin to fluoropyrimidine-based chemotherapy was associated with lower risk of death. Also, a

Table 3 Association between thymidylate synthase polymorphisms and patient characteristics

Polymorphism	Characteristic	RR (95%CI)	*P* value
2R	Age < 65-years-old	1.708 (1.158-2.520)	0.090
2RG/3RG	Grade 1-2	1.449 (1.077-1.948)	0.044
2RG/3RC	Female	1.943 (1.152-3.275)	0.036
ins/ins	*KRAS* G12D	3.563 (1.163-10.912)	0.045
3RG/3RC	*KRAS* wild-type	1.753 (1.156-2.657)	0.031

Table 4 Risk groups of thymidylate synthase polymorphisms

Group	Relapsed	*De novo* metastatic	Total
TYMS 5'UTR			
Low expression			
2RG	2 (4.2)	6 (14.6)	8 (9.0)
2RG/3RC	8 (16.7)	3 (7.3)	11 (12.4)
3RC	6 (12.5)	5 (12.2)	11 (12.4)
Medium expression			
2RG/3RG	4 (8.3)	4 (9.8)	8 (9)
2RG/3RCLOH	7 (14.6)	3 (7.3)	10 (11.2)
2RG/3RGLOH	3 (6.3)	2 (4.9)	5 (5.6)
2RGLOH/3RC	1 (2.1)	2 (4.9)	3 (3.4)
High expression			
3RG	4 (8.3)	4 (9.8)	8 (9.0)
3RG/3RC	9 (18.8)	8 (19.5)	17 (19.1)
2RGLOH/3RG	4 (8.3)	4 (9.8)	8 (9.0)
TYMS 3'UTR			
Low expression			
del/del	6 (12.5)	7 (17.1)	13 (14.6)
Medium expression			
ins/del	19 (39.6)	10 (24.4)	29 (32.6)
del/LOH	1 (2.1)	3 (7.3)	4 (4.5)
High expression			
ins/ins	8 (16.7)	13 (31.7)	21 (23.6)
ins/LOH	14 (29.2)	8 (19.5)	22 (24.7)

LOH: Loss of heterozygosity; UTR: Untranslated region.

statistical trend for a higher risk of death was shown in male patients. These findings were consistent in multivariate Cox regression analysis when the history of relapse or *de novo* metastatic disease was considered.

DISCUSSION

This is a retrospective study of 89 patients with mCRC treated with fluoropyrimidine-based chemotherapy, interrogating the association of *TYMS* polymorphisms, LOH, *KRAS*/*BRAF* status with survival outcome. To the best of our knowledge, this is the first time that *TYMS* genotype, LOH and mutations in *KRAS* and *BRAF* have been analyzed in relation to the chemotherapy treatment and survival outcome of patients with mCRC. We report that the polymorphisms of the *TYMS* 3'UTR represent an independent factor, increasing the risk for both disease progression and death of mCRC patients under fluoropyrimidines-based treatment as monotherapy or in combination with oxaliplatin or/and irinotecan, or/and targeted therapy. Also, an independent factor decreasing the risk of both

Table 5 Univariate Cox regression analysis for clinicopathological features and genotype

Variable	PFS			OS		
	HR	95%CI	*P* value	HR	95%CI	*P* value
KRAS mutated	1.390	0.895-2.164	0.142	1.669	0.996-2.797	0.052
BRAF V600E	0.884	0.356-2.196	0.791	1.514	0.545-4.207	0.426
LOH	1.013	0.632-1.624	0.957	1.020	0.592-1.758	0.944
TYMS 5′UTR			0.561			0.845
2R	1.000			1.000		
2R/3R	1.243	0.616-2.508	0.543	1.276	0.556-2.928	0.565
3R	0.974	0.474-2.003	0.944	1.239	0.535-2.870	0.616
TYMS 5′UTR			0.887			0.486
2RG	1.000			1.000		
2RG/3RC	1.151	0.535-2.475	0.720	0.978	0.388-2.468	0.963
2RG/3RG	1.351	0.625-2.921	0.444	1.688	0.689-4.132	0.252
3RC	1.038	0.428-2.517	0.935	0.876	0.293-2.620	0.813
3RG/3RC	0.883	0.391-1.995	0.764	1.648	0.660-4.113	0.284
3RG	1.107	0.433-2.832	0.832	1.054	0.348-3.189	0.926
TYMS 5′UTR			0.726			0.562
2R	1.000			1.000		
2RG/3RC	1.864	0.738-4.713	0.188	1.678	0.546-5.160	0.366
2RG/3RCLOH	0.783	0.300-2.044	0.617	1.044	0.328-3.323	0.942
2RG/3RG	1.058	0.372-3.014	0.916	1.869	0.537-6.504	0.325
2RG/3RGLOH	1.936	0.630-5.948	0.248	3.875	1.019-14.740	0.047
2RGLOH/3RC	1.155	0.301-4.441	0.834	1.091	0.126-9.412	0.937
2RGLOH/3RG	1.656	0.617-4.442	0.317	1.745	0.546-5.576	0.348
3RC	1.096	0.428-2.806	0.848	1.070	0.325-3.521	0.912
3RG	1.163	0.432-3.134	0.765	1.281	0.385-4.270	0.687
3RG/3RC	1.001	0.413-2.426	0.998	2.144	0.758-6.064	0.151
TYMS 5′UTR groups			0.812			0.489
Low expression	1.063	0.633-1.784	0.818	0.696	0.384-1.261	0.232
Medium expression	0.888	0.518-1.523	0.667	0.851	0.460-1.572	0.606
High expression	1.000			1.000		
TYMS 3′UTR			0.295			0.340
del/del	0.602	0.305-1.190	0.144	0.563	0.259-1.224	0.147
ins/del	0.764	0.475-1.228	0.267	0.910	0.522-1.587	0.739
ins/ins	1.000			1.000		
TYMS 3′UTR			0.067		1	0.095
del/del	0.421	0.194-0.912	0.028	0.311	0.125-0.772	0.012
del/LOH	0.784	0.263-2.334	0.662	0.773	0.175-3.417	0.734
ins/del	0.438	0.240-0.802	0.007	0.459	0.230-0.918	0.028
ins/LOH	0.516	0.274-0.973	0.041	0.488	0.233-1.020	0.057
ins/ins	1.000			1.000		
TYMS 3′UTR groups			0.225			0.187
Low expression	0.639	0.323-1.263	0.198	0.503	0.230-1.102	0.086
Medium expression	0.435	0.435-1.118	0.135	0.738	0.426-1.279	0.279
High expression	1.000			1.000		

CT: Chemotherapy; LOH: Loss of heterozygosity; OS: Overall survival; PFS: Progression-free survival; UTR: Untranslated region.

disease progression and death was the administration of fluoropyrimidine-based chemotherapy in combination with irinotecan, while the combination of fluoropyrimidines with oxaliplatin was associated with lower risk of death.

In search of prognostic markers towards personalized therapy, studies have investigated *TYMS* gene polymorphisms[30,31], *TYMS* mRNA expression[32,33] and TYMS protein expression[34-38]/activity[39]. Such studies have conflicting results for the way *TYMS* polymorphisms seem to affect the therapeutic result in CRC patients[30,36,38,40-46]. The numerous *TYMS* polymorphisms and their combination could explain the inconclusive results. For example, the SNP G>C was not considered for many years until its discovery[15,17,19]. Thus, the homozygous 3R group was considered to be related with high expression[15] and could include three subgroups with a different impact in *TYMS* expression (low expression subgroup 3RC/3RC and high expression subgroups 3RG/3RG and 3RG/3RC[24,28]).

Our results indicate that only 8 (21.6%) out of 37 tumors with the 3R polymorphism are 3RG, without the presence either of LOH or SNP G>C. Similarly, 21 (50%) out of 42 heterozygous 2R/3R tumors are 2RG/3RG. The different distribution of these subgroups in various studies could explain the differential effect on survival. Moreover, another factor held responsible for generating inconclusive results is the addition to fluoropyrimidines of newer chemotherapeutics and targeted agents[28] which incommode the interpretation of how *TYMS* polymorphisms influence survival outcome across different treatment populations. Moreover, there

Table 6 Multivariate Cox regression analysis

	PFS			OS		
	HR	95%CI	*P* value	HR	95%CI	*P* value
KRAS mutated	1.600	1.011-2.531	0.045			
LOH	1.674	0.912-3.071	0.096			
Fluoropyrimidine-based CT						
With irinotecan	0.600	0.372-0.969	0.037	0.352	0.164-0.757	0.007
Without irinotecan	1.000			1.000		
With oxaliplatin				1.000		
Without oxaliplatin				2.702	1.273-5.738	0.010
TYMS 3'UTR groups			0.043			0.027
Low expression	0.432	0.198-0.946	0.036	0.366	0.162-0.827	0.016
Medium expression	0.513	0.287-0.919	0.025	0.559	0.309-1.013	0.055
High expression	1.000			1.000		
Sex						
Males				1.580	0.916-2.724	0.100

CT: Chemotherapy; LOH: Loss of heterozygosity; OS: Overall survival; PFS: Progression-free survival; UTR: Untranslated region.

are other genes, such as *p53*[47], astrocyte elevated gene-1[48], and enolase superfamily member 1[49] that have been proven to participate in the final level of *TYMS* expression[18,47-50]. Thus, the rather small size samples used in most studies could not examine thoroughly the plethora of all these factors and possible interactions among them, without conflicting results.

Another reason responsible for conflicting results across studies is the categorization of *TYMS* polymorphisms into only two groups[18,51], which leads to misclassifying polymorphisms with uncertain effect. Although such a classification model is preferred because it facilitates statistical processing (*e.g.*, by increasing the size of each group) and the interpretation of statistical processing, it also entails the risk of increasing the probability of classification error.

Different to previous studies[30,31,50-53], ours took into consideration the extensive number of *TYMS* polymorphisms, as well as their combinations with LOH and *KRAS* / *BRAF* mutations. Additionally, for the first time, we classified the polymorphisms of each UTR region into three groups according to the level of *TYMS* expression.

The low expression group of 5'UTR polymorphism includes tumors with two alleles, each with one being an active USF-1 binding site. Members of the high expression group have no 2RG allele and they include heterozygous tumors in which, due to LOH, the allele 2RG was deleted. Medium expression group includes the heterozygous tumors with three USF-1 binding sites (one in the 2RG and two in the 3RG), resulting in one more than the low expression and one less than the high expression group. Also in this group, we included tumors with only one 2R allele, as LOH eliminates the 3R allele. Although they have less than three USF-1 binding sites, the LOH situation bears a loss of genetic material from chromosome 18q that, in ways not fully understood, adversely affects survival[54].

The low expression group of 3'UTR polymorphism contains the homozygous deletion of the 6 bp insertion that leads to destabilization of *TYMS* mRNA, resulting in reduced translation and eventually reduced TYMS activity. The high expression group has only ins alleles, homozygous or in combination with LOH, which impart stability to *TYMS* mRNA and thus, by increasing TYMS production/activity, increases the risk of poor response or development of resistance[18,19]. Tumors in the medium expression group have an allele with deletion, which coexists with either ins allele or LOH, that have been associated with increased risk of relapse[54].

On the basis of previous studies, *TYMS* 5'UTR may be linked to survival outcomes[41,55]. Contrary to these, in the multivariate Cox regression analysis of our data, the groups of 5'UTR polymorphisms did not emerge as factors of survival outcome. However, the 3'UTR polymorphisms' groups, were identified as independent factors of disease progression and death.

More specifically, the high expression group was identified as an independent risk factor of disease progression and death compared to the medium/low-risk groups (Table 5). Similar to our findings, a previous study showed that mCRC patients with del/del genotype treated with 5FU/oxaliplatin had significantly longer OS[31]. The ins allele, present in high-risk genotypes (ins/ins and ins/LOH) has been associated with higher *TYMS* mRNA stability and TYMS protein expression[18]. It is logical to assume that the mRNA stability has a more significant role in TYMS protein production than the number of transcripts. Hence, even if *TYMS* 5'UTR has a 3RG polymorphism leading to higher mRNA production, the complete absence of ins allele in *TYMS* 3'UTR could cause *TYMS* mRNA instability and therefore decreased TYMS translation. On the contrary, in theory the final outcome of decreased mRNA production of 2R cases combined with ins/ins genotype could be an increase of protein production due to the stability of transcribed mRNA and translational efficacy.

Tumors with 2R/3RLOH genotype have been shown

to be expressing significantly lower levels of TYMS protein than those with 2RLOH/3R[56]. Also, patients with mCRC bearing 2R/3RLOH genotype have been shown to have better survival than those with 2RLOH/3R[12]; although in the later study the SNP G>C was not taken into consideration. LOH is as likely to lead to altered genotypes, either with high or low TYMS protein expression (2RLOH/3RG and 2R/3RGLOH respectively). But the loss of chromosomal material from 18q, the cause of LOH, has been shown to act as a molecular marker of adverse prognosis[29], even if combined with the low-risk 2R allele. This is in agreement with our results as LOH remained in the Cox proportional hazards model as a factor that associates with disease progression with marginal statistical significance (HR = 1.674, 95%CI: 0.912-3.071, $P < 0.1$). This association was not observed for risk of death, probably due to the numerous factors that affect this outcome, such as the additional chemotherapy lines.

It has been previously shown that patients with *KRAS* mutant tumors had significantly lower *TYMS* mRNA levels, especially in proximal colon tumors[57]. In our study, we were able to identify an association of *KRAS* wild-type only with polymorphism 3RG/3RC (RR = 1.753, 95%CI: 1.156-2.657, $P < 0.04$), a member of the high TYMS protein expression group.

The addition of bevacizumab in the fluoropyrimidine-based 1st line chemotherapy for mCRC did not emerge, in the Cox model, as a factor affecting survival outcome in our study. To date, no prospectively validated biomarkers have emerged to include or exclude patients from anti-VEGF therapy[58]. Pander *et al*[59] have shown that there is a genetic interaction between the polymorphisms in the *TYMS* enhancer region (5'UTR) and VEGF +405g>c polymorphisms as a predictor of the efficacy of capecitabine/oxaliplatin/bevacizumab in mCRC patients, but only for PFS. Also, Watanabe *et al*[60] have found that higher TYMS levels are associated with an adverse response to bevacizumab therapy. In this context, it could be proposed that in studies applying anti-VEGF and targeted therapy, *TYMS* polymorphisms should be considered. Overall, there is great need for a prognostication model that would include all these polymorphisms with *RAS* mutations for treatment tailoring.

In our study, we did not examine TYMS protein expression, as this could be affected by a plentitude of factors[47,48,50] and altered in the course of the disease. For example, discordance in *TYMS* mRNA expression and TYMS protein levels has been found between primary and secondary tumors[33,61,62]. Also, in an autoregulatory manner, the binding of TYMS protein to its own mRNA, as well as the binding of TYMS to *p53* mRNA, causes translational repression[13,63,64].

Some limitations of this study should be addressed. The plethora of genotypes resulting from the polymorphisms occurring in the UTRs of *TYMS* is difficult to be analyzed with a small patient group. Moreover, previous exposure to adjuvant therapy with fluoropyrimidines, that could associate with resistance to fluoropyrimidines, was not taken into consideration. The allocation of *TYMS* polymorphisms into groups was based on published research but the conflicting results observed in these studies and ours highlight the need for further analysis on larger scale datasets. Also, we did not examine the TYMS protein expression and activity. Finally, due to the retrospective nature of this analysis we could not correlate these findings to the treatment toxicity.

After taking into account the SNP G>C and LOH, only the polymorphisms in the *TYMS* 3'UTR, affecting the stability of mRNA, independently influenced survival outcome for patients with mCRC treated with fluoropyrimidines-based chemotherapy. Genotypes that include del alleles, linked to *TYMS* mRNA instability, had better survival outcome. *KRAS* mutation was associated with high risk of disease progression. Combinations that included irinotecan were associated with lower risk of disease progression and death. Future studies should focus on gathering large samples and carefully selecting batteries of biomarkers to be examined in multivariate analysis. For the more complete assessment of the effect of *TYMS* gene polymorphisms, LOH should be considered. Further prospective studies are needed to elucidate the role of *TYMS* polymorphisms in tailoring treatment of patients with mCRC.

COMMENTS

Background

Metastatic colorectal cancer (mCRC) remains a significant cause of cancer-related death worldwide, although important improvements have been achieved in the last decades. It has been found that large variation exists in individual patient prognosis and response to chemotherapy, caused by molecular heterogeneity. Around 32%-40% of CRC harbor a *KRAS* mutation, which is a predictor of response to anti-EGFR treatment, while *BRAF* is a *KRAS* downstream abnormally activated kinase that has been shown to have similar adverse effects on treatment response. Several polymorphisms of the thymidylate synthase (*TYMS*) gene have been connected to variable TYMS protein levels and therapeutic outcome in relation to 5-FU, while loss of heterozygosity (LOH) is included in potential resistance mechanisms to fluoropyrimidines. This study aimed to investigate the associations of *TYMS* polymorphisms, LOH, *KRAS/BRAF* mutations and clinicopathologic characteristics with the survival outcome of patients with mCRC treated with 1st line fluoropyrimidine-based chemotherapy.

Research frontiers

To the best of the authors' knowledge, this is the first study that analyzes the extensive number of *TYMS* polymorphisms, particularly their combination with LOH and *KRAS* and *BRAF* mutations in relation to the chemotherapy treatment and the survival outcome of patients with mCRC. Additionally, for the first time, the authors classified the polymorphisms of each untranslated region (UTR) into three groups according to the level of *TYMS* expression. The results of this study contribute to clarifying the significance of *TYMS* polymorphisms for patients with mCRC.

Innovations and breakthroughs

In this study, the groups of *TYMS* 5'UTR polymorphisms did not emerge as factors of survival outcome. However, the 3'UTR polymorphisms' groups were identified as independent factors of disease progression and death. Genotypes

that included del alleles, linked to *TYMS* mRNA instability, had better survival outcome.

Applications

This study suggests that *TYMS* 3'UTR polymorphisms independently influence survival outcome for patients with mCRC treated with fluoropyrimidines-based chemotherapy. Genotypes that include del alleles may benefit from fluoropyrimidine-based chemotherapy. Future studies should gather large sample sets and carefully select the biomarkers to be examined in multivariate analysis, taking into consideration LOH.

Terminology

UTR: Regions of the mRNA that are not translated into protein but, among other things, affect the post-transcriptional regulation of gene expression. Upstream stimulatory factor: Factors that enhance the gene promoter and lead to increased gene expression.

Peer-review

Good overview of the role of TYMS in the treatment protocol. Will be of interest to the readership.

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