C677T and A1298C *MTHFR* gene polymorphisms and response to fluoropyrimidine-based chemotherapy in Mestizo patients with metastatic colorectal cancer

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Objective **To assess the association between C677T and A1298C methylenetetrahydrofolate reductase (*MTHFR*) single-nucleotide polymorphisms (SNPs) and response to first-line fluoropyrimidine-based chemotherapy for metastatic colorectal adenocarcinoma.**

Methods **A total of 68 patients were prospectively followed up in San Juan de Dios Hospital (San José, Costa Rica) from January 2019 to November 2020. Patients received first-line therapy with capecitabine or 5-fluorouracil in combination with oxaliplatin or irinotecan. Germline and somatic DNA was extracted from blood samples and paraffin-embedded tissue, respectively. Overall response rate (partial response + complete response) was assessed according to RECIST 1.1 criteria. Cox regression models were performed to identify the effect of *MTHFR* C677T and A1298C SNPs on progression-free survival (PFS) and overall survival (OS) (NCT registration number: 03852290).**

Results **Patients harboring one or both T alleles of the *MTHFR* C677T SNP had better overall response than homozygous wild-type individuals [odds ratio (OR): 3.21; 95% confidence interval (CI), 1.05–9.81; *P*=0.03]. No association was found between the *MTHFR* A1298C genotypes and overall response (OR: 0.75; 95% CI, 0.26–2.20; *P*=0.60). Patients with the *MTHFR* 677 TT and CT genotypes had longer PFS than CC individuals (hazard ratio: 0.53; 95% CI, 0.28–0.98; *P*=0.045), even after adjustment for confounders (hazard ratio: 0.50; 95% CI, 0.25–0.98; *P*=0.04). We found no association between the *MTHFR* A1298C SNP and PFS (hazard ratio: 1.35; 95% CI, 0.72–2.55; *P*=0.34). None of the SNPs was associated with OS.**

Conclusion **Patients carrying at least one mutant allele of the *MTHFR C*677T SNP had a better overall response and longer PFS than wild-type homozygous patients.**

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Keywords: colorectal neoplasms, Costa Rica, methylenetetrahydrofolate reductase, single-nucleotide polymorphism.

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Introduction

Colorectal cancer (CRC) is one of the leading causes of cancer-related death worldwide [1]. Despite novel therapies, the majority of patients with metastatic CRC die from their disease [2], highlighting the need for prognostic and predictive biomarkers to assess the risk of response, recurrence, and survival.

Fluoropyrimidines, such as capecitabine and its active metabolite 5-fluorouracil (5-FU), is the backbone of treatment for metastatic CRC, and are recommended therapies for these patients [3]. After cellular uptake, 5-FU is converted into 5-fluorouracil deoxynucleotide (5-FdUMP), which in combination with 5,10-methylenetetrahydrofolate (5,10-MTHF) inhibits thymidylate synthase (TS), an enzyme that catalyzes the conversion of uridine monophosphate to thymidine monophosphate [4–6]. This inhibition precludes the synthesis of purines, resulting in reduced DNA replication and repair [4–6]. In addition, 5-FU metabolites are largely incorporated into RNA molecules, leading to dysregulation of protein synthesis [5,6]. In these metabolic pathways, the methylenetetrahydrofolate reductase (MTHFR) plays a critical role in the irreversible conversion of 5,10-MTHF to 5-methyltetrahydrofolate, which is used as the substrate for methionine synthesis, the universal methyl donor for methylation of DNA, RNA, and proteins [7,8].

Two common single-nucleotide polymorphisms (SNPs), C677T (rs1801133; alanine to valine substitution at codon 222) and A1298C (rs1801131; glutamate to alanine substitution at codon 429) reduce MTHFR activity resulting in enhanced inhibition of TS, altered DNA methylation, and microsatellite instability (MSI) [7–11]. Previous studies have described that MTHFR activity is decreased by 70–75% in homozygous *MTHFR* 677 TT

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individuals compared to homozygous wild-types, and by 30–40% in homozygous *MTHFR* 1298 CC subjects, while heterozygous individuals exhibit intermediary activities [9,10]. It has been hypothesized that tumors exhibiting mutated *MTHFR* genotypes may be more sensitive to fluoropyrimidine-based chemotherapy than wild-type *MTHFR* tumors. Although some studies support this hypothesis [12–19], other trials have shown conflicting results [20–25]. These contradictory findings can be the result of different methodologies and techniques, or due to different chemotherapy regimens employed in each trial. Moreover, the prevalence of each of the aforementioned SNPs varies according to ethnicity [26,27], a fact that may further contribute to explain these contradictory findings.

Therefore, we carried out this study with the primary aim of determining the association between *MTHFR* polymorphisms (C677T and A1298C) and fluoropyrimidine-based chemotherapy response in a cohort of Mestizo patients with metastatic CRC from Costa Rica. Additionally, we explored if these SNPs were associated with clinical or molecular characteristics at diagnosis.

Materials and methods

Patients and clinical data

We performed a prospective study at Hospital San Juan de Dios, San José, Costa Rica (Caja Costarricense de Seguro Social). We included patients older than 18 years with a histologically confirmed diagnosis of stage IV colorectal adenocarcinoma who received first-line therapy with a fluoropyrimidine (capecitabine or 5-FU) in combination with either oxaliplatin or irinotecan. Eligible patients were required to have adequate organ function according to their attending oncologist, the life expectancy of more than 3 months, good performance status (ECOG performance status 0–2), and measurable disease according to the Response Evaluation Criteria in Solid Tumors (RECIST 1.1) [28]. Patients received one of the following chemotherapy schemes: (1) FOLFOX (5-FU 400 mg/m^2 as a bolus, plus leucovorin 400 mg/m^2 and oxaliplatin 85 mg/m^2 on day one; followed by 5-FU 2400 mg/m^2 as a 46-h continuous infusion every 15 days); (2) CAPEOX (capecitabine 1000 mg/m^2 twice daily for 14 days and oxaliplatin 130 mg/m^2 every 21 days); (3) FOLFIRI (5-FU 400 mg/m^2 as a bolus, plus leucovorin 400 mg/m^2 and irinotecan 180 mg/m^2 on day 1, followed by 5-FU 2400 mg/m^2 as a 46-h continuous infusion every 15 days). The aforementioned schemes were administered until disease progression or toxicity, according to the oncologist's criteria.

The RECIST 1.1 [28] was used to assess tumor response after 3 months of treatment initiation and every 3 months thereafter. The overall response rate (ORR) was defined as the proportion of patients that achieved a complete or partial response. Progression-free survival (PFS) was defined from the start date of systemic treatment to tumor progression per RECIST 1.1 criteria or death. Overall survival (OS) was defined as the period from treatment initiation to the date of death from any cause.

Ethical approval was obtained from the Institution's Ethics Committee (Comité Ético Científico Central, Caja Costarricense de Seguro Social, protocol no. R017-SABI-00126). All patients signed informed consents before inclusion (NCT: 03852290).

Blood and tumor tissue processing

Before treatment initiation, venous blood samples were collected into tubes containing 1.7 mM EDTA and stored at –20°C until testing. Germline DNA was extracted automatically using the QIAamp DNA Blood Mini Kit with QIAcube (Qiagen, Valencia, California, USA). Formalin-fixed paraffin-embedded (FFPE) tissues were macrodissected after hematoxylin-eosin staining and slide identification of tumor content by an experienced pathologist. Somatic DNA from FFPE was extracted using the QIAamp DNA FFPE Tissue Kit (Qiagen), according to the manufacturer's recommendations.

DNA quality was evaluated by Nanodrop 260/280 and 260/230 ratios (Thermo Fisher Scientific, Wilmington, Delaware, USA) and concentration was quantified with a Qubit Fluorometer (Life Technologies, Carlsbad, California, USA) according to the manufacturer's recommendations.

Assessment of MTHFR polymorphisms

The *MTHFR* C677T and A1298C SNPs were determined by PCR – restriction fragment length polymorphism analysis. For the *MTHFR* C677T polymorphism, the sequences of primers were 5′-TGAAG GAGAAGGTGTCTGCGGGA-3′ and 5′-AGGACGGT GCGGTGAGAGTG-3′. The PCR products were amplicons of 198 bp and were digested with 1 unit of Hinf I for 8 h. For the *MTHFR* A1298C polymorphism, the primer sequences were 5′-CTTTGGGGAGC TGAAGGACTACTAC-3′ and 5′-CACTTTGTGACCA TTCCGGTTTG-3′. The PCR products were amplicons of 163 bp and were digested by 1 unit of Mbo II. The PCR was performed in a thermal cycler (Veriti, Foster City, California, USA, Applied Biosystem) using 50–100 ng/sample and PCR conditions were an initial preheating step of 95°C for 3 min, followed by 30 cycles of 95°C for 1 min, an annealing step at 55°C for 1 min and an extension step at 72°C for 1 min. The last step of an extension was performed at 72°C for 7 min. Regarding the C677T SNP, since mutation creates a Hinf I restriction site, the amplicon of the wild-type allele (198 bp) is not cut, while the mutant allele is cut into two fragments of 175 and 23 bp, respectively. The A1298C mutation abolishes Mbo II restriction site, therefore, the amplicon of the wild-type allele showed fragments of 56, 30, 28, and 19 bp, while the mutant allele only showed three segments of 84, 30, and 19 bp. The digested PCR products were separated on GelRed (Biotium, San Francisco, California, USA) dye stained 3% agarose gels and visualized under ultraviolet

light. Positive control was included in all the electrophoresis samples for comparative purposes.

Assessment of *KRAS*, *NRAS*, and *BRAF* mutations

For the assessment of 29 mutations of *KRAS*, 22 mutations of *NRAS*, and 9 mutations of *BRAF*, we used the *KRAS*, *NRAS*, and *BRAF* StripAssays (ViennaLab, Vienna, Austria) according to the manufacturer's instructions. It detects the *KRAS* mutations G12A, G12R, G12D, G12C, G12I, G12L, G12S, G12V, G13D, G13C, G13A, G13R, G13D, G13C, G13S, G13V, A59E, A59G, A59T, G60V, G61R, G61H, Q61L, Q61K, K117N, K117E, A146P, A146T, and A146V; the *NRAS* mutations G12A, G12R, G12D, G12C, G12S, G12V, G13R, G13D, G13C, G13V, A59D, A59T, G60R, G60E, Q61R, Q61E, Q61L, Q61K, Q61P, A146T and the *BRAF* mutations V600E (c. 1799 T>A and c.1799_1800delTGinsAA), V600A, V600D, V600G, V600K, V600M, V600R, and K601E. A PCR enriched for mutant *KRAS*, *NRAS*, and *BRAF* alleles were performed. This PCR is based on wild-type sequence clamping with a specific PNA oligonucleotide, allowing preferred amplification of the mutant sequence. Subsequently, PCR products were hybridized to a nitrocellulose strip containing specific probes for the different mutations. After hybridization, the test strip was washed, blocked, and color was developed.

Assessment of microsatellite instability status

The Idylla MSI Assay (Biocartis, Mechelen, Belgium) was performed as described previously [29]. Briefly, macrodissected FFPE tumor samples were loaded into an Idylla MSI cartridge and inserted into the console following the manufacturer's instructions. The Idylla MSI Assay is an automated in-vitro diagnostic test intended for the qualitative detection of seven monomorphic biomarkers (*ACVR2A*, *BTBD7*, *DIDO1*, *MRE11*, *RYR3*, *SEC31A*, and *SULF2*) for the identification of microsatellite status in CRC. Once the FFPE tissue is inserted in a single-use cartridge, nucleic acids are liberated by a combination of enzymatic/chemical digestion, high-intensity focused ultrasound, and heat. Then, the specific targets are detected using fluorescently labeled molecular beacons after PCR amplification. A fluorophore-based detection system monitors the differential melting behavior of wild-type and mutant amplicons with automated algorithms. The MSI software automatically checked the validity of the measured fluorescence profiles and provides the final report. Tumors were defined as having MSI-H if at least two of the seven MSI markers were positive, and MSS (microsatellite stability) if it did not meet these criteria.

Statistical analysis

Categorical variables are presented as frequencies and compared by the chi-square or Fisher's test when applicable. Continuous variables are expressed by means and SD and compared through the analysis of variance tests. Genotype distributions were tested for agreement with those expected under the Hardy-Weinberg equilibrium (HWE) using the chi-square test. The crude odds ratio (OR) of ORR was calculated with its 95% confidence interval (CI). The probability of PFS and OS were calculated using the Kaplan–Meier method, measuring time from the date of first treatment to the date of progression or death, as recorded by the Costa Rican Civil Registry. Cases were censored at the last clinical follow-up in November 2020. Comparisons between genotypes were done under a dominant model (TT + CT vs. CC for the *MTHFR* C677T and AC + CC vs. AA for the *MTHFR* A1298C) through the Log-rank test. A univariate and multivariate Cox proportional hazard regression model was performed to identify variables associated with PFS. Those variables with a *P* value less than 0.2 were selected in a stepwise fashion for inclusion into the multivariate model. A univariate logistic regression analysis was done to calculate the OR of response according to genotype. A *P* value less than 0.05 was considered statistically significant. Analyses were performed with the SPSS software version 21.0 for Mac (Chicago, USA).

Results

Clinical characteristics

A total of 68 patients were included in the study. Overall clinical characteristics and categorized according to the genotypes of both *MTHFR* SNPs are summarized in Table 1. The majority of patients were female ($n=35$, 51.5%) with good performance status at treatment initiation. Most patients had left-sided or rectal primary tumors ($n=54$, 79.4%) that were usually removed before therapy ($n=39$, 57.4%). Synchronous metastatic disease with liver or lung involvement was the most frequent clinical presentation ($n=52$, 76.5%). No clinical characteristic was associated with any of the *MTHFR* genotypes. Besides, we found no association neither for the correlation between these polymorphisms and MSI, nor for the presence of *KRAS*, *NRAS*, and *BRAF* mutations.

Genotyping

C677T *MTHFR* genotypic frequencies were distributed as follows: CC ($n=18$, 26.5%), CT ($n=34$, 50%), and TT ($n=16$, 23.5%). The T allele frequency was 49.5%, following the HWE ($P=0.99$). A1298C *MTHFR* genotypic frequencies were AA ($n=48$, 70.5%), AC ($n=16$, 23.5%), and CC ($n=4$, 6%). The C allele frequency was 24%, also following the HWE ($P=0.11$). No patients were found to be homozygous for both loci.

Concordance of genotypes from EDTA-blood (germline) and FFPE (somatic) was 93.7%, corresponding to a number of discordant pairs of 4.

Impact of MTHFR polymorphisms on overall response rate

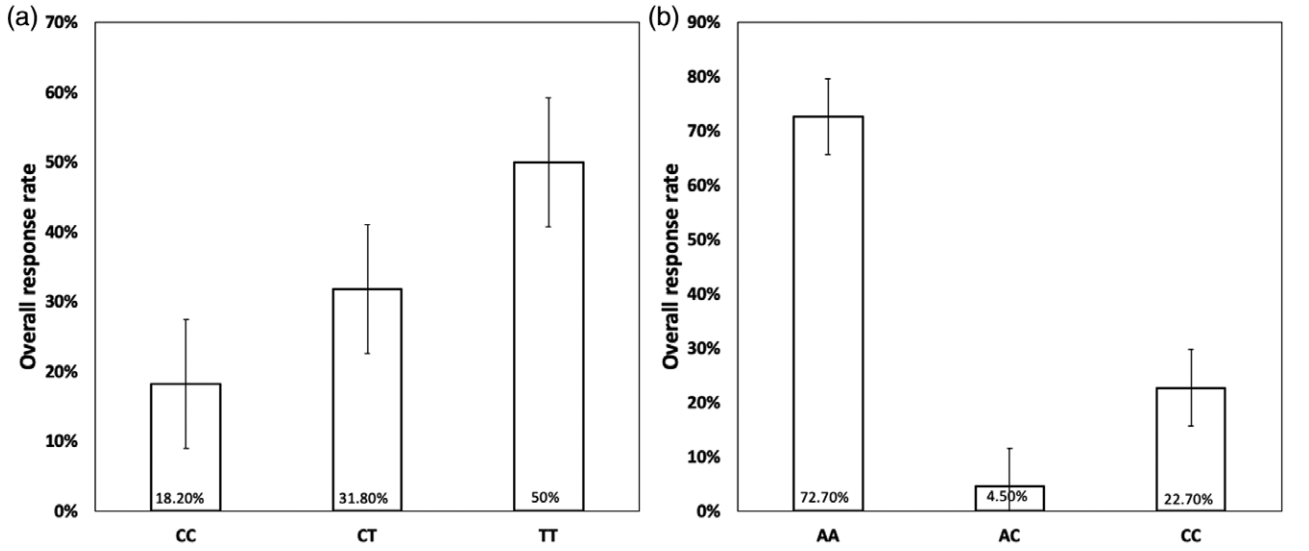
Patients harboring the *MTHFR* 677 TT and CT genotypes had better ORR than wild-type patients (Fig. 1a).

Table 1 Clinical characteristics of the studied population

Variable	Overall (*n*=68)	*MTHFR* C677T			*P* value*	*MTHFR* A1298C			*P* value*
		CC (*n*=18)	CT (*n*=34)	TT (*n*=16)		AA (*n*=48)	AC (*n*=16)	CC (*n*=4)	
Age (years±SD)	59.4±13.7	61.0±14.8	57.4±12.5	61.9±15.1	0.47	59.7±13.6	57.4±13.9	63.5±16.9	0.71
Sex (%)					0.91				0.09
Female	35 (51.5)	9 (50)	17 (50)	9 (56.2)		27 (56.2)	8 (50)	0	
Male	33 (48.5)	9 (50)	17 (50)	7 (43.8)		21 (43.8)	8 (50)	4 (100)	
Performance status (ECOG) (%)					0.11				0.50
0	42 (61.8)	8 (44.4)	25 (73.5)	9 (56.2)		31 (64.6)	8 (50)	3 (75)	
1	26 (38.2)	10 (55.6)	9 (26.5)	7 (43.8)		17 (35.4)	8 (50)	1 (25)	
Primary tumor location (%)					0.21				0.69
Right colon	14 (20.6)	4 (22.2)	5 (14.7)	5 (31.2)		11 (22.9)	3 (18.7)	0	
Left colon	28 (41.2)	9 (50)	14 (41.2)	5 (31.2)		17 (35.4)	8 (50)	3 (75)	
Rectum	26 (38.2)	5 (27.8)	15 (44.1)	6 (17.6)		20 (41.7)	5 (31.3)	1 (25)	
Pathological tumor stage (T) (%)					0.69				0.49
II	1 (1.5)	0	1 (2.9)	0		1 (2.1)	0 ()	0	
III	35 (51.5)	11 (61.1)	17 (50)	7 (43.8)		27 (56.2)	7 (43.7)	1 (25)	
IV	20 (29.4)	6 (33.3)	9 (26.5)	5 (31.2)		12 (25)	7 (43.7)	1 (25)	
Unknown	12 (17.6)	1 (5.6)	7 (20.6)	4 (25)		8 (16.7)	2 (12.6)	2 (50)	
Pathological nodal status (N) (%)					0.73				0.34
0	12 (17.6)	3 (16.7)	7 (20.6)	2 (12.5)		10 (20.8)	1 (6.1)	1 (25)	
I	34 (50)	11 (61.1)	16 (47.1)	7 (43.8)		22 (45.8)	11 (68.7)	1 (25)	
II	10 (14.7)	3 (16.7)	4 (11.7)	3 (18.7)		8 (16.7)	2 (12.6)	0	
Unknown	12 (17.6)	1 (5.6)	7 (20.6)	4 (25)		8 (16.7)	2 (12.6)	2 (50)	
Microsatellite instability (%)	8 (11.7)	2 (11.1)	5 (14.7)	1 (6.3)	0.74	5 (10.4)	3 (18.7)	0	0.72
Mutation status (%)					0.67				0.81
KRAS	16 (23.5)	4 (22.2)	8 (23.5)	4 (25)		10 (20.8)	5 (31.3)	1 (25)	
NRAS	22 (32.4)	6 (33.3)	12 (35.3)	4 (25)		14 (29.2)	7 (43.8)	1 (25)	
BRAF	7 (10.3)	2 (11.1)	4 (11.8)	1 (6.3)		6 (12.6)	1 (6.1)	0	
Metastasis (%)					0.51				0.49
Metachronous	16 (23.5)	5 (27.8)	6 (17.6)	5 (31.3)		13 (27.1)	2 (12.5)	1 (25)	
Synchronous	52 (76.5)	13 (72.2)	28 (82.4)	11 (68.7)		35 (72.9)	14 (87.5)	3 (75)	
Site of metastasis (%)					0.78				0.86
Liver	38 (55.9)	10 (55.6)	20 (58.8)	8 (50)		26 (54.2)	9 (56.3)	3 (75)	
Lung	23 (33.8)	7 (38.9)	10 (29.4)	6 (37.5)		15 (31.2)	7 (43.8)	1 (25)	
Lymph nodes	18 (26.5)	6 (33.3)	8 (23.5)	4 (25)		11 (22.9)	6 (37.5)	1 (25)	
Peritoneum	17 (25)	3 (16.7)	9 (26.5)	5 (31.2)		13 (27.1)	3 (18.7)	1 (25)	
Central nervous system	1 (1.5)	0	1 (2.9)	0 ()		1 (2.1)	0	0	
Primary tumor resected (%)	39 (57.4)	11 (61.1)	18 (52.9)	10 (62.5)	0.76	29 (60.4)	8 (50)	2 (50)	0.73
Metastatic lesion resected (%)	30 (44.1)	9 (50)	12 (35.3)	9 (56.3)	0.32	21 (43.7)	7 (43.7)	2 (50)	0.97
Chemotherapy scheme (%)					0.31				0.83
FOLFOX	31 (45.6)	9 (50)	15 (44.2)	7 (43.8)		21 (43.7)	7 (43.8)	3 (75)	
CAPEOX	25 (36.8)	6 (33.3)	17 (50)	4 (25)		18 (37.5)	6 (37.5)	1 (25)	
FOLFIRI	12 (17.6)	3 (16.7)	4 (11.8)	5 (31.2)		9 (18.8)	3 (18.7)	0	

CAPEOX, capecitabine and oxaliplatin; ECOG, Eastern Cooperative Oncology Group; FOLFIRI, 5-fluorouracil, leucovorin, and irinotecan; FOLFOX, 5-fluorouracil, leucovorin, and oxaliplatin.
*The *P* value corresponds to the comparison between genotypes.

Fig. 1



Overall response rate according to the *5,10-methylenetetrahydrofolate reductase* (*MTHFR*) 6C77T (a) and A1298C (b) polymorphisms.

The OR of achieving complete or partial response for patients with at least one mutant T allele was 3.21 (95% CI, 1.05–9.81; $P = 0.03$). No association was found between the *MTHFR* A1298C polymorphisms and response to chemotherapy with an OR (AC + CC vs. AA) of 0.75 (95% CI, 0.26–2.20; $P = 0.60$) (Fig. 1b).

Impact of MTHFR polymorphisms on progression-free and overall survival

At the time of analysis, with a median follow-up of 20.7 months, a total of 42 patients had progressed and 29 subjects had died. Patients with at least one mutated allele of the *MTHFR* C677T polymorphism exhibited longer PFS than homozygous wild-type patients (Fig. 2a). Median PFS was 17.3 months for CT and TT individuals vs. 13.7 months for CC subjects (hazard ratio = 0.53; 95% CI, 0.28–0.98; $P = 0.045$). There was no association between the *MTHFR* A1298C polymorphism and PFS (hazard ratio = 1.35; 95% CI, 0.72–2.55; $P = 0.34$) (Fig. 2b).

The univariate and multivariate analyses for PFS and OS are shown in Table 2. Only the resection of the primary tumor and the presence of at least one mutant allele for the *MTHFR* C677T polymorphism were independent variables for PFS.

Survival models by Kaplan–Meier estimates and Cox proportional regression analyses found no significant association between the two studied *MTHFR* SNPs and OS. Only the ECOG performance status and the primary tumor resection were independent variables associated with this outcome.

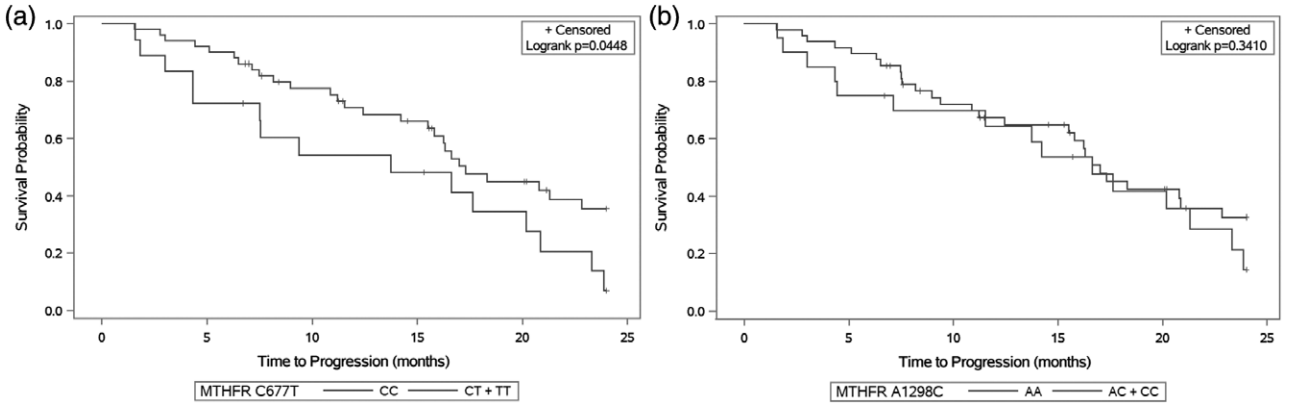
Discussion:

The findings of this prospective cohort suggest that patients carrying at least one T allele of the *MTHFR* C677T polymorphism exhibit a greater response and longer PFS than wild-type individuals. However, our results did not show any significant association between the aforementioned SNP and OS, nor for the interaction between the *MTHFR* A1298C polymorphism and the efficacy outcomes of clinical response, PFS, or OS. Besides, we did not find any clinical or molecular characteristics correlated with any of these SNPs.

Our findings are in accordance with previous studies regarding the influence of *MTHFR* genotypes on tumor response [12–19,30]. For instance, Jakobsen and colleagues [30] reported that patients with the *MTHFR* 677 TT genotype achieved greater response rates and longer time to progression than homozygous wild-type patients. Similarly, Ettiene-Grimaldi *et al.* [12] reported that mutant alleles of both *MTHFR* SNPs (677T and 1298C) were positively linked to response in a cohort of patients that were treated with FOLFOX regimen. Furthermore, other authors have reached analogous findings, not only in the assessment of response and PFS but also regarding an OS improvement among metastatic patients carrying one or both mutant alleles of the *MTHFR* C677T polymorphism [30]. All these conclusions have been validated in pre-clinical models with human cancer cell lines [31,32] and, in conjunction with clinical data, support the hypothesis of increased sensitivity to 5-FU in individuals with low activity of the MTHFR [7–10].

Despite our findings and the aforementioned studies have shown a positive impact on tumor response according to the *MTHFR* C677T polymorphism, other authors have published conflicting or nonsignificant results [20–25,33,34]. In a recent meta-analysis, Chen and colleagues [22] did not observe any significant association between

Fig. 2



Progression-free survival (Kaplan–Meier method) according to the *5,10-methylenetetrahydrofolate reductase* (*MTHFR*) C677T (a) and A1298 (b) genotype.

patients carrying the mutant T allele of the *MTHFR* 677 polymorphism and PFS or OS. In contrast, they found that patients with the CC genotype of the *MTHFR* A1298C had significantly poorer outcomes than wild-type individuals. Similarly, no significant association between the previous *MTHFR* SNPs and PFS was reported by Ruzzo and coworkers in patients treated with either FOLFOX [24] or FOLFIRI [25] for metastatic CRC.

The discrepancies between our findings and other studies can be the result of distinct treatment regimens. It has been argued that the mechanism of action of 5-FU varies depending on the mode of administration. Continuous infusion exerts its major effect on TS inhibition, whereas bolus 5-FU may have a preferential role influence on RNA [6]. Of note, the infusion regimen employed by Ruzzo and colleagues [24] was lower than the 5-FU dose administered in our cohort. Another source of variation is related to the addition of leucovorin to 5-FU. It has been stated that leucovorin, a precursor of MTHF, can enhance the inhibition of TS by stabilizing the ternary complex of 5,10-MTHF, 5-FdUMP, and TS. In the negative study of Marcuello and collaborators [34], almost half of patients did not receive this drug.

Previous reports with inconsistent findings have also described that some source of variation can arise if the determination of *MTHFR* polymorphisms is performed in somatic or germline DNA [21]. However, we found an excellent agreement between pairs of genotypes from both peripheral blood and FFPE samples, as previously suggested by other authors [35]. Therefore, other sources of heterogeneity must be more relevant to explain these opposing results.

One major difference between our data and previous studies with opposite findings resides in the ethnic background of each studied population. Indeed, the frequency of the *MTHFR* 677T allele in our study was higher than that reported in some of the studies with diverging results (49.5% vs. 28 [26] to 33% [23]). Therefore, these conflicting studies may lack statistical power to detect significant differences among patients according to their genotype. In contrast, the multivariate analysis carried out in our study (Table 2) confirms the independent value of the *MTHFR* C677T SNP to predict PFS.

Although we did not detect any association between the A1298C SNP and tumor response, PFS, or OS, the low prevalence of the *MTHFR* 1298 C allele in our cohort hinders the precision of these results. Regarding this potential association, some authors have also reported inconclusive findings [18,36]. Once again, the source population of each trial, the nonuniform treatment protocols and schedules, and the retrospective design of some analyses, with their inherent limitation of controlling for confounders, can explain the inconsistent clinical evidence.

The results from this report are of particular interest for our Region due to the relatively high prevalence of the 677T allele among Mestizo populations, with reported frequencies from 49.5% (in our cohort) to 57% in Mexican individuals [19,26]. Indeed, the mutant allelic frequency of both *MTHFR* SNPs was very similar to that described for Latin American populations and lower than that reported Caucasian patients [27]. These differences support the fact that distinctive outcomes can be observed among patients treated with 5-FU-based chemotherapy, depending on the genetic background of each specific population.

Our data did not find any significant association between the studied *MTHFR* SNPs and OS. However, we consider that PFS is a more valid outcome to assess the clinical impact of both SNPs and response to chemotherapy, since OS might be influenced by selective use of second-line

Table 2 Univariate and multivariate models for progression-free and overall survival

Variable	Progression				Death			
	Univariate model		Multivariate model		Univariate model		Multivariate model	
	Hazard ratio (95% CI)	*P* value	Hazard ratio (95% CI)	*P* value	Hazard ratio (95% CI)	*P* value	Hazard ratio (95% CI)	*P* value
Sex								
Female	1 (reference)				1 (reference)			
Male	0.98 (0.54–1.81)	0.96			1.03 (0.50–2.13)	0.93		
Age								
≥65 years	1 (reference)				1 (reference)			
<65	0.66 (0.45–1.67)	0.66			0.79 (0.34–1.85)	0.58		
ECOG Performance status								
1-2	1 (reference)		1 (reference)		1 (reference)		1 (reference)	
0	0.44 (0.24–0.81)	0.009*	0.57 (0.30–1.08)	0.08	0.28 (0.13–0.58)	0.001*	0.34 (0.16–0.75)	0.007*
Primary tumor resection	0.43 (0.23–0.79)	0.006*	0.49 (0.25–0.97)	0.04*	0.31 (0.15–0.66)	0.003*	0.43 (0.19–0.98)	0.046*
Timing of metastastatic disease								
*S*ynchronous	1 (reference)				1 (reference)			
Methacronous	0.74 (0.37–1.48)	0.39			0.69 (0.31–1.50)	0.34		
Metastatectomy	0.64 (0.35–1.19)	0.16	0.69 (0.36–1.33)	0.27	0.61 (0.29–1.28)	0.19	0.88 (0.40–1.95)	0.88
MTHFR C677T								
CC	1 (reference)		1 (reference)		1 (reference)			
CT + TT	0.53 (0.28–0.98)	0.045*	0.50 (0.25–0.98)	0.04*	0.86 (0.38–1.94)	0.71		
MTHFR A1298C								
AA	1 (reference)				1 (reference)			
AC + CC	1.35 (0.72–2.55)	0.34			1.24 (0.58–2.67)	0.58		

CI, confidence interval.*Statistically significant at $P<0.05$.

and third-line treatments, with or without target therapies, loco-regional treatments, and deaths not attributed to cancer. Nevertheless, a longer follow-up of our cohort, with more mature data will determine the real effect of these genetic markers on this particular outcome.

Our study has some limitations due to the relatively small sample size and the short follow-up period. Furthermore, we did not test for other relevant genetic variants involved in fluoropyrimidine metabolism, such as those affecting the function of the dihydropyrimidine dehydrogenase (DPYD) and TS, that have also been related to the toxicity and efficacy of this therapy [4,37]. Indeed, the variant allele frequency in similar population databases varies broadly, ranging from 0.14% (for the *DPYD* rs3918290 variant) [38], to 65% (for the *TS* rs34743033 polymorphism) [39]. Similarly, another potential source of bias can be attributable to different chemotherapy schemes used in the first-line setting that could affect the efficacy outcomes. However, we consider this effect very unlikely since previous studies have shown that these schemes have comparable response rates, PFS, and OS [40]. Therefore, despite the aforementioned caveats, we consider our data to provide a valid approach to identify genetic predictive markers of fluoropyrimidine-based chemotherapy efficacy in a specific population of Mestizo ethnicity.

It has been proposed that *MTHFR* polymorphisms can affect methylation patterns of DNA repair genes, resulting in MSI [11]. However, our data did not support this assumption. We consider that this contradictory finding can be the result of the low percentage of MSI tumors found in our cohort since larger studies have confirmed this theory [11,41].

Although analysis of somatic mutations (such as *KRAS*, *NRAS*, and *BRAF*) and detection of MSI are now applied in clinical practice to distinguish patients who will benefit from anti-EGFR and immunotherapy, respectively, more predictive factors are needed in order to predict the response to fluoropyrimidines, that still remains the cornerstone of treatment for metastatic CRC treatment. Therefore, our data contribute to add more evidence on this potential role of genetic biomarkers in a specific population, usually underrepresented from large clinical trials.

In conclusion, our findings support the role of the *MTHFR* C677T SNP as a genetic predictive variable of tumor responsiveness and an independent prognostic factor for PFS in patients with metastatic CRC that are treated with fluoropyrimidine-based chemotherapy. Larger studies with Mestizo populations are warranted to confirm our results.

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Conflicts of interest

There are no conflicts of interest.

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