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ORIGINAL ARTICLE

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Methylenetetrahydrofolate reductase gene polymorphisms: genomic predictors of clinical response to fluoropyrimidine-based chemotherapy?

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Abstract Fluorouracil (5-FU) is widely used in the treatment of colorectal cancer. Methylenetetrahydrofolate reductase (MTHFR) may play a central role in the action of 5-FU, an inhibitor of thymidylate synthase, by converting 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. The aim of this study was to ascertain whether two polymorphisms in the MTHFR gene (677C > T and 1298 A > C) could be used as genomic predictors of clinical response to fluoropyrimidine-based chemotherapy (in combination with irinotecan or oxaliplatin). Ninety-four patients diagnosed with metastatic colorectal cancer and undergoing 5-FU-containing chemotherapy as a first line treatment were studied. The results suggest that the MTHFR genotype cannot be considered as an independent factor of outcome in colorectal cancer patients under 5-FU-based chemotherapy.

Keywords Colon cancer · Fluoropyrimidines · Methylenetetrahydrofolate reductase · Pharmacogenetics

Introduction

A high percentage of colorectal cancer, which is a leading cause of cancer-related morbidity and mortality in the industrialized world, is diagnosed at advanced stages when chemotherapy is required for its management.

5-Fluorouracil (5-FU), a drug of choice for the treatment of patients with metastatic colorectal cancer, exerts its anticancer effect through the inhibition of thymidylate synthase (TS) [10]. TS catalyzes a critical reaction in cell proliferation: the methylation of dUMP to dTMP with 5,10-methylenetetrahydrofolate (5, 10-methyleneTHF) as the methyl donor [2] (Fig. 1). TS inhibition occurs when a stable ternary complex between the 5-FU metabolite (fluorodeoxyuridine monophosphate, FdUMP), the enzyme and the methyl donor is formed. Optimal TS inhibition requires elevated cellular concentrations of 5,10-methyleneTHF, and this can account for the fact that clinical studies have demonstrated higher antitumor efficacy when 5-FU is associated with folinic acid (FA), a precursor of 5,10-methyleneTHF.

The intracellular concentration of 5,10-methyleneTHF is controlled by the activity of methylentetrahydrofolate reductase (MTHFR), an enzyme that plays a key role in the metabolism of folate. This enzyme catalyzes the irreversible conversion of 5,10-methyleneTHF to 5,10- methyltetrahydrofolate (5,10-methylTHF). Polymorphisms in the MTHFR gene (677C > T and 1298 A > C) associated to a decreased enzyme activity would result in higher concentrations of 5,10-methyleneTHF that would in turn favor the formation and stability of the inhibitory ternary complex involving TS, 5,10-methyleneTHF and FdUMP. Thus, patients with mutated alleles should be more sensitive to 5-FU than patients with a wild-type genotype.

The MTHFR gene is located on chromosome 1p, and is composed of 11 exons [6]. The best characterized MTHFR polymorphism consists of a 677C > T transition, in exon 4, which results in an alanine to valine substitution in the predicted catalytic domain of MTHFR. This substitution renders the enzyme thermolabile, and homozygotes and heterozygotes have about 70% and 35% reduced enzyme activity, respectively [5]. A second common polymorphism in the MTHFR gene is a 1298 A > C transition in exon 7,

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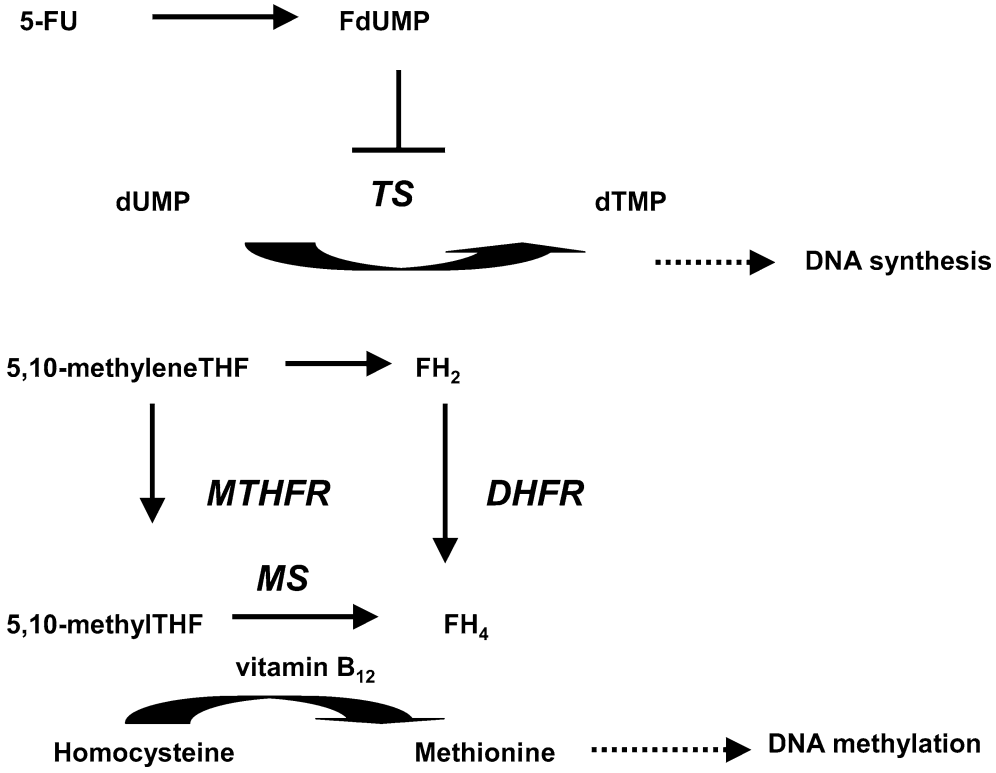

Fig. 1 Simplified metabolic pathways of folate metabolism

which results in a glutamate to alanine substitution within a presumed regulatory domain of the protein [17]. The 1298C allele leads to a decreased enzyme activity, although not to the same extent as the 677T allele. Individuals who are compound heterozygous for the 677T and 1298C alleles have a 40–50% reduced MTHFR activity and a biochemical profile similar to the one observed among 677T homozygotes.

If a decreased MTHFR activity favors thymidine synthesis via an increase of 5,10-methyleneTHF, cancer patients with MTHFR genotypes associated with enzymatic deficiency may be more sensitive to 5-FU chemotherapy than patients with a wild-type genotype.

The aim of the present work was to ascertain whether the MTHFR functional polymorphisms are predictors of clinical response to 5-FU- based chemotherapy in a cohort of 94 patients. Moreover, we sought to determine whether they enhance the predictive value of TS polymorphisms, recently demonstrated in this group of advanced colorectal cancer patients [11].

Materials and methods

Patients

Ninety four patients diagnosed with metastatic colorectal cancer and undergoing 5-FU-containing chemotherapy as a first line treatment were studied. All patients were required to have normal bone marrow and organ function before administration of 5-FU. The exclusion criteria was ECOG > 3. All patients gave written informed consent, and the study was approved by the Institutional Ethics Committee.

Chemotherapy regimen description

The three different regimens administered in this group of patients were:

- Regimen A: A dose of 2,250 mg/m^2 of 5-FU (in continuous infusion for 48 h i.v.) and irinotecan (80 mg/m^2 infused in 45 min i.v.) every week.
- Regimen B: 5-FU (a pulse dose of 400 mg/m^2 on days 1 and 2 and a continuous infusion for 44 h of 1,200 mg/m^2) and irinotecan (180 mg/m^2 i.v.), every 2 weeks.
- Regimen C: 5-FU (at the same dose as in regimen B), leucovorin and oxaliplatin (85 mg/m^2 infused for 2 h) every 2 weeks.

Patients underwent chemotherapy cycles until severe toxicity or disease progression (DP) appeared.

Given that regimens A and B contained similar doses of 5-FU and irinotecan administered in accordance with different schedules and given that they did not include leucovorin, the two regimens were considered as one group in the statistical analysis.

Clinical parameters

Relevant clinical data were obtained from clinical records (gender, age, ECOG, colon vs rectal involvement,

previous adjuvant therapy, multifocal vs single metastasis). Response to treatment and overall survival as were also analyzed. Clinical response was assessed (CT scan) 3 months after the start of chemotherapy. Complete remission (CR) was defined as the disappearance of tumor masses and disease-related symptoms and as the normalization of the initially abnormal tests and/or biopsies for at least 1 month. Partial remission (PR) was considered when measurable lesions decreased by at least 50%. Objective response (OR) was assumed when a CR or PR was obtained. Patients without criteria of clinical response but without progression were regarded as patients with stable disease (SD). DP during or after treatment was also taken into account. OS was calculated from the start of chemotherapy to death regardless of the cause. Progression free survival (PFS) was calculated from the beginning of the therapy to the time of DP, relapse or death [19].

Genotyping

After receiving informed consent, we obtained EDTA-whole blood from 94 patients, and DNA was isolated by the salting out procedure [13]. The SNPs MTHFR 677 and MTHFR 1298 were detected by means of real-time PCR on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Primers and TaqMan probes were as previously described [9]. Each reaction contained template DNA and a final concentration of 1× TaqMan PCR Master Mix (Applied Biosystems), 300 nM of each primer, 100 nM of wild-type probe (Applied Biosystems) and 100 nM of variant probe (Applied Biosystems). Thermocycling was performed with an initial 50°C incubation for 2 min followed by a 10 min incubation at 95°C. A 2-step cycling reaction was performed for 40 cycles, with denaturation at 95°C for 15 s and annealing/extension at 55°C for 1 min. Analysis of the amplification reaction was performed using the Sequence Detector software, version 2.0 (Applied Biosystems).

Statistical analysis

Differences between the categorical variables were measured by the Chi-square test. Logistic regression was used as a multivariate method to ascertain whether the MTHFR genotype independently predicted OR. The model was adjusted to the other clinically relevant variables (age, gender, performance status, tumor localization, previous adjuvant therapy, single or multifocal metastasis, chemotherapy regimen and TS genotype). Kaplan Meier estimates and log-rank tests were employed in the univariate analysis of OS and PFS. Cox regression methods were used for the OS and PFS multivariate analysis. The results were considered to be statistically significant when bilateral P values were less than 0.05.

Results

Clinical data and allelic frequencies

A total of 94 patients who fulfilled all inclusion and exclusion criteria were studied. Their clinical data are given in Table 1.

The frequency of the MTHFR 677T allele was 74/188 = 0.4 (95%CI; 0.3–0.5). We detected 12 T/T homozygous and 50 C/T heterozygous patients. The frequency of the MTHFR 1298C allele was 58/188 = 0.3 (95%CI; 0.2–0.4). Eight patients were C/C homozygous and 42 were A/C heterozygous.

Correlation between MTHFR genotypes and the outcome of chemotherapy

Clinical response was assessable in all patients. Five CR, 44 PR, 30 SD and 15 tumor progressions were observed, yielding an OR rate of 57%.

In the univariate analysis between the MTHFR mutations and response, no relationship was found between presence of C677T mutation ($P = 0.7$). A trend to a better outcome of homozygous A1298C patients was detected ($P = 0.1$) (Tables 2, 3).

Table 1 Clinical data

Age
Median: 68 years
Range: 43–83 years
Sex
Males: 68 (72%)
Females:26 (28%)
ECOG
< 2:83 (88%)
≥2: 11 (12%)
Site
Colon:64 (68%)
Rectum:30 (32%)
Previous adjuvant therapy
Yes:31 (33%)
No: 63 (67%)
Metastasis
Single:71 (75%)
Multifocal: 23 (25%)
Chemotherapy treatment
A:17 (18%)
B:19 (20%)
C:58 (62%)

Table 2 Relationships between tumor responsiveness and MTHFR gene polymorphisms

Genotype	CR	PR	SD	PROGR
677 T/T	1	4	4	3
677 C/T	3	23	16	8
677 C/C	1	17	10	4
1298 C/C	0	7	1	0
1298 A/C	2	17	19	4
1298 A/A	3	20	10	11

Table 3 Objective responses according to the MTHFR genotype

	MTHFR-677	MTHFR-1298
(A)		
Homozygous mutant	5/12 (41.7%)	7/8 (87.5%)
Heterozygous	26/50 (52%)	19/42 (45.2%)
Wild-type	18/32 (56.3%)	23/44 (52.3%)
P	0.7	0.1
		Objective response
(B)		
MTHFR genotype associated with a decreased enzymatic activity[a]		24/47 (51.1%)
MTHFR genotype associated with an increased enzymatic activity[b]		25/47 (53.2%)
P		1

[a](Homozygous 677T, homozygous 1298C and compound heterozygous patients)
[b](Heterozygous and wild-type patients)

Table 4 Objective responses according to the MTHFR genotype and Leucovorin (LV) administration

	LV +	LV−	*P*
MTHFR genotype associated with a decreased enzymatic activity	12/24 (50%)	12/23 (52%)	0.9
MTHFR genotype associated with an increased enzymatic activity	7/12 (58%)	18/35 (51%)	0.8
P	0.7	1	

In an additional univariate analysis, we compared the clinical outcome of these patients with MTHFR genotypes associated with a low (homozygous and compound heterozygous patients) or with a high (heterozygous and wild-type patients) enzymatic activity. No relationship was observed ($P = 1$) (Table 3).

Furthermore, we sought to find out whether the administration of leucovorin modified the clinical outcome in this group of patients in accordance with the above mentioned MTHFR genotype. Table 4 shows that there are no significant differences in the percentage of patients achieving an OR between groups.

We investigated the independent predictor value to obtain a clinical response of genotypes associated with low/high MTHFR activity. A logistic regression model including these genotypes and all relevant clinical variables—such as gender, age, ECOG, chemotherapy treatment, colon versus rectal involvement, previous adjuvant therapy, multifocal versus single metastasis, chemotherapy regimen and TS genotype—was used. The MTHFR genotype showed no predictive value ($P = 0.7$).

Correlation between genotypes and survival

No differences in PFS or OS were found between patients homozygous or compound heterozygous for the two MTHFR mutations when compared with the group of heterozygous or wild-type patients ($P = 0.8$ and $P = 0.7$ respectively) (Fig. 2).

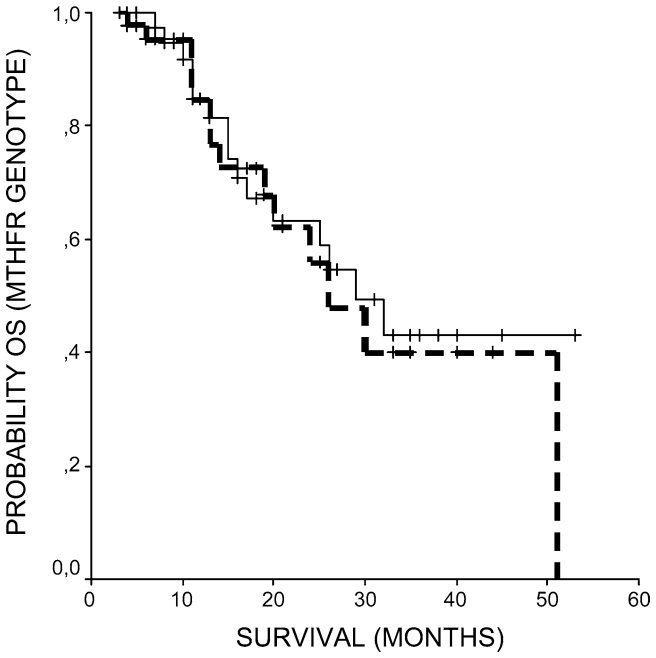

Fig. 2 Kaplan Meier estimates of overall survival according to MTHFR genotypes (homozygous and double heterozygous patients in *continuous line* and heterozygous and wild type patients in *dotted line*). The statistical significance was 0.8

In a Cox regression model, MTHFR mutations were not independently significant to predict PFS or OS ($P = 0.9$ and 0.3, respectively) after adjustment to the other clinically relevant variables (Table 5).

Table 5 Cox regression analysis of PFS (A) and OS (B)

Variable	Coeff B	*P*	RR
A			
MTHFR Genotype	0.017	0.9	1.02
TS Genotype	0.62	0.04	1.9
Age	0.008	0.6	1
Gender	−0.37	0.3	0.7
Ecog	0.6	0.1	1.8
Chemotherapy type	−0.2	0.7	0.8
Previous adjuvant therapy	−0.2	0.6	0.8
Colon vs rectal localization	0.4	0.2	1.5
Single vs multifocal metastasis	0.7	0.05	1.9
B			
MTHFR Genotype	0.4	0.3	1.5
TS Genotype	0.9	0.05	2.4
Age	0.1	0.003	1.1
Gender	−0.45	0.3	0.6
Ecog	0.3	0.6	1.3
Chemotherapy type	−0.8	0.17	0.4
Previous adjuvant therapy	−0.06	0.9	0.9
Colon vs rectal localization	0.02	0.96	1
Single vs multifocal metastasis	1.3	0.01	3.8

Discussion

5-FU exerts its antineoplastic effect by inhibition of TS. This inhibition is mediated by the formation of a ternary complex between the enzyme (TS), the drug (FdUMP) and the methyl donor (5,10-methyleneTHF). The intracellular concentration of this last compound is controlled by the activity of the enzyme MTHFR.

Two functional SNPs (C677T and A1298C) have been described in the MTHFR gene. The mutated forms of these variants exhibit lower enzymatic activity and should lead to a high level of intracellular 5,10-methyleneTHF that will favor the formation and stability of the inhibitory ternary complex involving TS, 5,10-methyleneTHF and FdUMP. It can be, therefore, hypothesized that patients with mutated alleles should be more sensitive to 5-FU than patients with a wild-type genotype.

Frequencies for these polymorphisms show marked ethnic and geographic variations. In Europe, there appears to be an increasing frequency of the variant form of the C677T from North to South [18]. In Spain, the overall frequency of homozygosity for the T allele is 12% [12], similar to that reported in the Mediterranean countries. For A1298C, a prevalence of 6% CC subjects has been reported in Spain [7], within the range found in other European studies [17]. The allelic frequencies detected in our patients resemble those in the aforementioned Spanish population.

In two recent experimental publications [14, 16] it has been demonstrated that antisense-mediated down-regulation of the MTHFR gene results in a reduced cancer cell survival in vitro, as well as a decrease of tumor growth in the human colon and lung xenografts, in vivo. These results suggest that MTHFR may be required for tumor cell survival and that MTHFR inhibition should be considered for antitumor therapy.

There have been few ''in vitro'' studies on the relation between MTHFR polymorphisms and chemosensitivity to 5-FU, yielding contradictory results. The use of the human colon and breast cancer cells transfected with wild-type or mutant 677T human MTHFR cDNA provides evidence that the MTHFR C677T polymorphism affects the concentration and intracellular distribution of folates and changes the growth and chemosensitivity of colon and breast cancer cells [15]. According to these authors, this polymorphism could be an important pharmacogenetic determinant of 5-FU and methotrexate-based cancer chemotherapy. The study conducted by Etienne et al. [3] on a panel of 19 human cancer cell lines reported a greater 5-FU efficacy in mutated MTHRF variants in position 1298 compared to wild-type homozygous cell lines.

In the first clinical study [1], the relationship between the C677T polymorphism and response to fluoropyrimidine-based chemotherapy in 43 patients with metastatic colorectal adenocarcinoma was determined. The authors found a statistically significant difference in the frequency of the T allele among responders versus non-responders and an odds ratio of 2.86 for a response in individuals with a T allele.

Etienne et al. [4] evaluated the MTHFR polymorphisms in 98 patients with metastatic colorectal cancer treated with 5-FU and leucovorin. The response rate was not related to 1298 A > C genotype but was significantly linked to 677 C > T genotype (response rate: 40%, 21% and 56% in CC, CT and TT, respectively; $P = 0.04$).

In a similar study performed by Jakobsen et al. [8], the response rate for 677 TT was 66% compared with 33% and 21% for 677 CC and 677 CT, respectively ($P = 0.04$). No correlation with response was observed in the case of 1298 A > C polymorphism.

In the present work we found no correlation between the MTHFR genotype and the outcome of chemotherapy. In the univariate analysis, no relationship was found between the presence of C677T mutation and the clinical response. Only a trend to a better outcome of homozygous 1298C patients was found (seven out of eight patients with this genotype achieved a clinical response). Also, we did not find any significant differences in the clinical outcome between these patients with MTHFR genotypes associated with low (homozygous and compound heterozygous patients) or with high (heterozygous and wild-type patients) enzymatic activity. The MTHFR genotype showed no predictive value for a clinical response in a logistic regression model. This lack of predictive value is not modified by folate supplementation, an additional finding reinforcing the ''in vitro'' results obtained by Ettienne et al. [3].

Our results showed no correlation between MTHRF polymorphisms and survival. No differences in PFS or OS were found between patients homozygous or compound heterozygous for the two MTHFR mutations when compared with the group of heterozygous or wild-type patients. In the Cox regression model, MTHFR mutations were not independently significant to predict PFS or OS after adjustment to the other clinically relevant variables.

The three clinical studies mentioned above [1, 4, 8] suggested that C677T genotyping could have predictive value in patients treated with fluoropyrimidine drugs. Our results are not consistent with this conclusion. Relevant differences between the chemotherapy regimens used in the published clinical studies and those used in our set of patients could account for these contradictory results. These authors included in their studies patients treated with monochemotherapy regimens (5FU/LV) whereas our patients followed a combined chemotherapy scheme (5-FU/irinotecan in 36 cases and 5-FU/oxaliplatin in 58 cases). An ideal model to establish the relationship between MTHFR mutations and response would be the study of patients treated with a single drug, but this situation is extremely uncommon in clinical practice, where combined chemotherapy regimens are mainly used. In the latter scenario, patients in whom the lack of response to 5-FU is MTHFR

genotype-dependent could respond to the effect of an additional drug. This would mask the correlation found by Cohen et al. [1], Ettienne et al. [4] and Jakobsen et al. [8] in their studies of cancer patients treated with a single antineoplastic agent. Our results reflect the lack of predictive value of the MTHFR genotype in cancer patients under combined chemotherapy regimens.

In the present work, we considered the TS genotype (including both the VNTR and the SNP located within the promoter region of the gene) in all multivariate studies. This variable remained as a significant independent predictor of clinical response, PFS and OS. Of particular significance is the fact that these TS polymorphisms predict response to 5-FU in combination with other drugs (irinotecan and oxaliplatin) currently used for the treatment of colorectal cancer.

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