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MTHFR polymorphisms and 5-FU-based adjuvant chemotherapy in colorectal cancer

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Background: Methylenetetrahydrofolate reductase is a pivotal enzyme in folate metabolism and 5-fluorouracil (5-FU) cytotoxicity. Two common single-nucleotide polymorphisms (SNPs), *MTHFR* 677C>T (rs1801133) and 1298A>C (rs1801131), reduce enzyme activity. Initially, these SNPs were claimed to predict clinical efficacy, but further studies have yielded contradictory results. We tested whether these two polymorphisms are determinants of clinical outcome in a large patient group with a long follow-up time.

Patients and methods: We included 331 patients who had been treated with adjuvant 5-FU/leucovorin chemotherapy after intended curative resection between 1997 and 2003. Clinical data, including relapse rates, overall survival, and tumor stage, were collected. DNA was extracted from formalin-fixed tumor tissue and analyzed for the *MTHFR* 677C>T and 1298A>C SNPs with real-time PCR.

Results: The *MTHFR* 677C>T and 1298A>C polymorphisms were not associated with survival or relapse-free survival ($P > 0.2$). The 677 *CC* genotype was associated to toxicity (odds ratio = 1.83, $P = 0.01$).

Conclusions: The *MTHFR* 677C>T and 1298A>C polymorphisms probably do not predict efficacy of adjuvant 5-FU treatment in colorectal cancer after complete resection; however, the 677C>T polymorphism may be associated with lower toxicity in 5-FU treatment. Implementation of SNP analysis for these polymorphisms for individualized treatment is premature.

Key words: colorectal cancer, 5-fluorouracil, methylenetetrahydrofolate reductase, pharmacogenetics, single-nucleotide polymorphisms

introduction

Colorectal cancer (CRC) has a worldwide incidence of 1 million new cases annually and claims the lives of half a million people every year. Curative surgery is attempted at the time of diagnosis for 75% of patients, but up to 50% of all patients will develop incurable recurrences making the 5-year survival rate only 50% [1].

For 30 years, the mainstay of adjuvant therapy has been 5-fluorouracil (5-FU). 5-FU has several cytotoxic mechanisms among which the inhibition of the enzyme thymidylate synthase (TYMS) is to be the most important. TYMS catalyzes the conversion of deoxyuridylate (dUMP) to deoxythymidylate (dTMP), in which the folate derived cofactor 5,10-methylenetetrahydrofolate (5,10-MTHF) functions as a methyl donor. 5-FdUMP, a metabolite of 5-FU, in combination with 5,10-MTHF inhibits TYMS in an almost irreversibly forming a ternary complex [2–5]. The lack of intracellular dTMP leads to decreased DNA synthesis, dUMP misincorporation into DNA, and DNA strand breaks followed by cell apoptosis [6, 7]. Experiments have proved that increased intracellular concentrations of reduced folates enhances 5-FU efficacy [2, 4].

Early clinical studies showed that 5-FU-containing regimes had a beneficial effect on survival [8]; later combinations of 5-FU with leucovorin (LV, [6R,S]5-formyltetrahydrofolate) were proven superior to other 5-FU regimes [9–11].

It has been hypothesized that reduced activity of methylenetetrahydrofolate reductase (MTHFR) could cause an increased intracellular concentration of 5,10-MTHF and augment the cytotoxicity of 5-FU. MTHFR is a pivotal enzyme in folate metabolism that catalyzes the irreversible conversion of 5,10-MTHF to 5-methyltetrahydrofolate, the latter is used in methionine synthesis (and consequently in DNA methylation) and the former is used in nucleotide synthesis.

The *MTHFR* gene is located on chromosome 1p36.3 and is subject to several single-nucleotide polymorphisms (SNPs). Two common SNPs cause reduced enzyme activity in

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homozygous individuals: the *MTHFR* 677C>T polymorphism induces an Ala-to-Val substitution in the catalytic domain (70% reduction in activity), whereas the *MTHFR* 1298A>C polymorphism induces a Glu-to-Ala substitution in a regulatory domain (30%–40% reduction) [12–14]. Compound heterozygous individuals have a 40%–50% reduction in enzyme activity [13, 14].

Various experimental [15, 16] and clinical studies [17–28] have been carried out to test the association between *MTHFR* polymorphisms and 5-FU treatment effect.

Hitherto available studies focus on two patient populations (Table 1); those undergoing treatment for metastatic colorectal cancer [17–20, 22–25, 28] and those undergoing adjuvant therapy before or after surgery [21, 26, 27]. Some studies show a positive effect on survival or response/relapse rates for the 677T allele [17–20], others show no effect of *MTHFR* genotype [21–25], and others again show a negative effect of 677T or 1298C alleles [19, 26–28]. Some studies suggest that the correlation between *MTHFR* polymorphisms and 5-FU treatment efficacy may only be significant for specific subgroups such as rectal cancer patients [26] or women [28], but this remains to be confirmed.

We have tested the effect of *MTHFR* genotype on 5-FU/LV treatment of one of the largest patient samples to date. Our patient sample has only received monotherapy with 5-FU/LV as adjuvant treatment that allows us to determine the effect of genotype on survival and relapse rates specifically during 5-FU/LV treatment.

patients and methods

patients

A total of 331 Caucasian patients with stage II–III and 37 patients with a singular excised liver metastasis from colorectal cancer (stage IV), treated at Rigshospitalet (Copenhagen University Hospital) from 1996 to 2003, were eligible for our study. The patients were recruited for testing of molecular prognostic/predictive markers in adjuvant treatment of colorectal cancer. The protocol allowed for collection of formalin-fixed paraffin-embedded (FFPE) tissue.

Patient data were entered and were followed in a database that was updated automatically every 6–12 months with relapse and death rates

Table 1. Overview of previous studies of MTHFR polymorphisms and colorectal cancer

Study	*n* (patients)	SNP	Patient group	Treatment	Result
Wisotzkey et al.	51	677C>T	Stage III (adjuvant)	5-FU/LV	No effect (survival)
Cohen et al.	43	677C>T	Metastatic	5-FU/LV or tegafur or capecitabine	T-allele associated with better response
Etienne et al.	98	677C>T; 1298A>C	Metastatic	5-FU/LV (three different regimes)	677C>T: no effect; 1298A>C: mutant homozygous had shorter survival
Jakobsen et al.	80 and 48 (retro- and prospective)	677C>T; 1298A>C	Metastatic	5-FU/LV	677C>T: mutant homozygous had better response rates; 1298A>C: no effect
Marcuello et al.	94	677C>T; 1298A>C	Metastatic	5-FU with irinotecan; 5-FU with oxaliplatin	No effect (response and survival)
Suh et al.	53	677C>T	Metastatic	FOLFOX	No effect (response and survival)
Terrazino et al.	122	677C>T; 1298A>C	Rectal cancer patients: T3–T4 and/or node positive	Radiotherapy and 5-FU alone or 5-FU/LV or 5-FU with oxaliplatin or 5-FU with carboplatin	677C>T: T-allele associated with worse response; T-A haplotype: associated with worse response
Ruzzo et al.	146	677C>T; 1298A>C	Metastatic	FOLFIRI	No effect (progression-free survival)
Ruzzo et al.	166	677C>T; 1298A>C	Metastatic	FOLFOX	No effect (progression-free survival)
Capitain et al.	76	677C>T; 1298A>C	Metastatic	5-FU/LV	TYMS 3R/3R with homozygous wild-type alleles for 677C>T or 1298A>C is associated with shorter survival
Zhang at al.	303	677C>T; 1298A>C	Metastatic	5-FU/LV; 5-FU/irinotecan; 5-FU/oxaliplatin	1298A>C: Homozygous mutants associated to shorter survival in female patients
Lurje et al.	197	677C>T; 1298A>C	Stage II and III (adjuvant)	5-FU/LV	No effect (time to tumor recurrence)

MTHFR, methylenetetrahydrofolate reductase; SNP, single-nucleotide polymorphism; 5-FU, 5-fluorouracil; LV, leucovorin.

through a central cancer and death registry (that includes cause of death). The latter is possible since every person in Denmark has a central personal registry number that is used for every registration in the whole of Denmark. Clinical data and data regarding tumor stage characteristics were collected retrospectively through chart review and reviewing of the tumor samples. Last follow-up date was 30 August 2007. Entry date was the date of (first) curative resection.

All patients were treated by intended curative surgery followed by adjuvant therapy with 5-FU/LV. The adjuvant treatment was the Mayo regimen, including bolus infusion of 5-FU (425 mg/m^2) and isovorin (10 mg/m^2) for 5 days, repeated every 4 weeks, for six cycles. None of the patients had been pretreated with any chemotherapeutic regime. This study was approved by the local ethics committee (protocol H-KF-01-201/03, amendment 18370).

genotyping

Tumor sections were selected from blocks containing approximately 50% tumor tissue.

Three 10 μm sections of each FFPE tumor tissue block were deparaffinized using Estisol 220 and genomic DNA was extracted using the Maxwell™ 16 Tissue DNA purification Kit on a Maxwell™ 16 instrument (Promega, Denmark) according to the manufacturer's instructions. A NanoDrop ND1000 spectrophotometer (Nanodrop Technologies Inc., Rockland, DE) was used to assess DNA quantity.

DNA samples were diluted to 10 ng per well and analyzed for the *MTHFR* 677C>T (rs1801133) and 1298A>C (rs1801131) polymorphisms using the fluorogenic 5′-nuclease assay (TaqMan® SNP Genotyping Assay Made to Order on an ABI 7900 HT, Applied Biosystems, Foster city, CA).

Genotypes were determined in a reaction mix as follows: 25 μl containing 10 ng DNA, 14 μl primer/probe mix with TaqMan Universal PCR Master mix (Applied Biosystems) according to the manufacturer's instructions.

PCR amplification was carried out with an initial step of 95°C for 10 min followed by 40 cycles of 92°C for 15 s and 60°C for 1 min (Applied Biosystems 7900HT Sequence Detection System). The fluorescence profile of each well was measured in an Applied Biosystems 7900HT Sequence Detection System, and the results were analyzed with Sequence Detection Software (SDS 2.3, Applied Biosystems).

Controls were included on each plate. Reproducibility was checked by regenotyping ∼10% of the cases.

statistical analysis

The primary end points of this study were overall survival (OS), relapse-free survival (RFS), and toxicity. Relapse was defined as the recurrence of disease or death from disease whatever occurred first. OS and RFS were measured from the date of intended curative resection to the date of relapse or death from disease (CRC-related deaths as deaths were actually all due to disease). Patients who were alive or had not relapsed at the last follow-up (30 August 2007) were censored at that time. Toxicity was measured according to the Common Toxicity Criteria (CTC) (National Cancer Institute—Common Toxicity Criteria version 2.0, www.fda.gov/ cder/ cancer/toxicityframe.htm) and maximum toxicity score was used in the statistical analysis.

The associations of clinicopathological characteristics (age, sex, tumor stage, vascular and perineural invasion, tumor grade, bowel obstruction and perforation, and rectal or colon tumor) and MTHFR polymorphisms with RFS and OS were assessed using univariate survival analysis (Kaplan–Meier curves and the log-rank test).

Multiple regression analysis of the independent effects of the two *MTHFR* polymorphisms on OS and RFS was carried out using the Cox Proportional Hazards model. Assumptions for the Cox analysis were checked for each variable; Cox analysis was only carried out if the variable fulfilled the assumptions. Some variables were registered during treatment (toxicity and performance) contrary to the other variables that were available at entry date. These three variables were only analyzed in regard to OS and RFS in patients alive after completion of treatment (180 days after entry date). Interactions between polymorphisms and the aforementioned clinicopathological variables on RFS and OS were tested by comparing likelihood ratio statistics with or without inclusion of the multiplicative product terms.

The association between MTHFR polymorphisms and overall toxicity was tested using univariate logistic regression and logistic regression analysis adjusted for sex, performance status, days to relapse, and age [29, 30]. Further subgroup analysis of toxicity was carried out according to CTC for: infections, cardiotoxicity, diarrhea, fatigue, mucositis, and nausea/ vomiting.

The associations of *MTHFR* polymorphisms and clinicopathological factors were analyzed using contingency tables and Fisher's exact tests were suitable.

Linkage disequilibrium between the two *MTHFR* polymorphisms using D′ and r2, haplotype frequencies, and Hardy–Weinberg (HW) equilibrium was calculated using Haploview v4.0 (www.broad.mit..edu/mpg/haploview/).

All reported *P* values are two sided. All analyses were carried out using the SAS Statistical Package Version 9.1.3 (SAS Institute Inc, Cary, NC) and Statistica 7.0 software (Statsoft Inc. Tulsa, OK).

results

clinical and pathological characteristics

Patient characteristics are summarized in Table 2. Median age was 61 years; the male to female ratio was 1 : 1. Clinically, intestinal perforation was present in 27 cases and bowel obstruction occurred in 49 cases at surgery. There was a follow-up time of up to 10 years and 169 (51%) had relapsed and 149 (45%) had died at the last follow-up date.

Clinically, performance score, tumor stage, and bowel obstruction were associated to OS and RFS, whereas toxicity was only associated to RFS (Table 3). Vascular and perineural invasion were also associated to OS and RFS in univariate analysis.

In multiple regression analysis adjusted for covariates, performance score, tumor stage, bowel obstruction, and vascular invasion were associated with OS. Performance score, tumor stage, bowel obstruction, toxicity, and perineural invasion were associated with RFS at the $P < 0.05$ level (Table 3). Tumor stage was not associated with OS and RFS when stage IV patients were excluded from analysis, indicating a similar prognosis for stage II and III patients.

MTHFR polymorphisms were not associated with any of the abovementioned clinical or pathological characteristics except toxicity and tumor site. The MTHFR677 TT genotype was overrepresented among rectal cancer patient (11 of 63 patients) compared with colorectal cancer patients (17 of 268) ($P = 0.01$), such an association was not found for the MTHFR1298 polymorphisms.

MTHFR 677C>T and 1298A>C polymorphisms

MTHFR genotypes were determined for 312 and 309 of 331 cases for the 677C>T and 1298A>C polymorphisms, respectively. Both genotypes were obtained for 290 of 331 cases as the DNA yield or quality was too low in remaining cases. We

Table 2. Demographic, clinical, and pathological information

Characteristic	Total patients *n* (%)
Age at diagnosis	
≤60	162 (49)
>60	169 (51)
Sex	
Women	165 (50)
Men	166 (50)
Tumor site	
Colon	268 (81)
Rectum	63 (19)
Stage	
II	36 (11)
III	258 (78)
IV	37 (11)
Tumor grade	
Undescribed	4 (1)
Low	98 (30)
High	91 (27)
Medium	138 (42)
Perineural invasion	
Undescribed	99 (30)
Present	63 (19)
None	169 (51)
Vascular invasion	
Undescribed	88 (27)
V0	173 (52)
V1	70 (21)
Intestinal perforation	
Present	27 (8)
Not present	304 (92)
Bowel obstruction	
Present	49 (15)
Not present	282 (85)
Performance	
0	179 (54)
1	112 (34)
2	25 (8)
3	8 (2)
4	7 (2)
Death	
Censored	182 (55)
Events	149 (45)
Relapse	
Censored	162 (49)
Events	169 (51)

found that 10% were 667C>T or 1298A>C homozygous (Table 4), which is concordant with earlier studies [12–14, 17–29, 31]. Both genotype distributions were in HW equilibrium and reproducibility was 100% for our genotyping.

We analyzed the effect of the *MTHFR* 677C>T and 1298A>C polymorphisms on OS and RFS after stratification according to genotype both using three groups (MUT/MUT versus MUT/WT versus WT/WT) and two groups (MUT/MUT versus MUT/WT and WT/WT). We tested subgroups to see if there was an association between genotype and treatment response stratified according to tumor site, sex, tumor stage, tumor grade, or vascular/neuronal invasion. No association was found using Kaplan–Meier curves and the log-rank test in analysis of OS and RFS.

Associations between OS and RFS and the *MTHFR* polymorphisms are summarized in Figure 1 and Table 3. Multiple regression analysis of OS and RFS showed similar results after adjusting for covariates (Table 3). Each clinical variable was checked for interaction with the polymorphisms using the Cox analysis; none was found.

When adjusting for perineural and vascular invasion, we can only analyze a subset of patients due to missing data. Since there is no logical reason why the missing data should introduce systematic bias into our data, we think it is justified to carry out the analysis. Interestingly, excluding perineural and vascular invasion from the Cox model does not change anything regarding polymorphism associations or model fit statistics.

Logistic regression showed that grade 3 and 4 toxicity was overrepresented among patients with the *MTHFR* 677 CC genotype with an odds ratio (OR) 1.83 [95% confidence interval (CI) 1.13–2.96, $P = 0.01$]. The association remained significant after adjusting for the aforementioned factors associated with toxicity (OR = 1.83, 95% CI 1.10–3.03, $P = 0.01$). Subgroup analysis revealed that the 677 CC genotype was particularly associated with grade 3–4 infections (OR = 4.57, 95% CI 1.28–16.38, $P = 0.02$) and diarrhea (OR = 1.98, 95% CI 1.07–3.65, $P = 0.03$). After adjustment, the association with infections was borderline significant (OR = 3.90, 95% CI 1.00–15.17, $P = 0.05$), whereas the association with diarrhea remained significant (OR = 1.99, 95% CI 1.07–3.69, $P = 0.03$). The MTHFR 1298A>C polymorphism was not associated with any toxicity.

haplotype analysis

There was a strong linkage disequilibrium between the two polymorphisms, in accordance with previous reports [29], with a D′ of 0.97 but r^2 was only 0.2. The three most common haplotypes (C-A, T-A, and C-C) accounted for 99.7% of all haplotypes. None of the haplotypes was associated with OS or RFS; not in the total sample or in any subgroups analyzed.

discussion

The main result of this study is that isolated SNPs in *MTHFR* probably cannot be used to predict the effect, or rather possible therapeutic failure, of adjuvant 5-FU treatment in colorectal cancer patients. However, we found a statistically significant association between the *MTHFR* 677C>T polymorphism and toxicity in accordance with earlier studies [29]. Furthermore, the univariate and the multiple regression analysis showed toxicity to be an independent predictor of relapse and death. These findings have to be interpreted with caution and could be the result of multiple testing; applying the Bonferroni correction yields a significance level of 0.005, but this procedure is notorious for being overly conservative.

Subgroup analysis of sex and other clinical variables did not reveal any associations as reported earlier [26, 28].

Table 3. Summary of statistically significant and genotype associations with OS and RFS

		Relapse				Death			
		Univariate analyses		Multiple regression (adjusted for covariates)		Univariate analyses		Multiple regression (adjusted for covariates)	
Characteristic		Median days to relapse	Survival curves, *P* value	Hazard ratio (95% CI)	*P* value	Median days to death	Survival curves, *P* value	Hazard ratio (95% CI)	*P* value
677 C>T	CT/CC	2231	0.69	1.10 (0.64–1.88)	0.73	2957	0.85	1.01 (0.56–1.84)	0.97
	TT	1338				–			
1298 A>C	AC/AA	2244	0.56	1.15 (0.71–1.86)	0.56	–	0.38	1.25 (0.76–2.05)	0.39
	CC	2272				2272			
Tumor stage	II	2272	<0.0001	2.31 (1.49–3.58)	0.0002	–	<0.0001	1.79 (1.13–2.85)	0.01
	III	2957				–			
	IV	436				929			
PNI	Yes	818	0.0006	1.58 (1.03–2.41)	0.03	1451	0.001	1.52 (0.96–2.39)	0.07
	No	–				–			
VI	Yes	767	0.0003	1.49 (0.97–2.30)	0.07	1423		1.61 (1.02–2.55)	0.04
	No	–				–			
Bowel obstruction	Yes	655	<0.0001	2.33 (1.40–3.88)	0.001	1215	<0.0001	2.15 (1.27–3.63)	0.005
	No	2848				–			
Performance	0–2	2791	<0.0001	3.44 (1.58–7.47)	<0.0001	–	<0.0001	3.20 (1.40–7.28)	<0.0001
	3–4	287				362			
Toxicity (CTC)	0–2	–	0.03	1.49 (1.06–2.10)	0.02	–	0.14	1.35 (0.93–1.95)	0.12
	3–4	1492				2957			

OS, overall survival; RFS, relapse-free survival; CI, confidence interval; PNI, perineural invasion; VI, vascular invasion; CTC, Common Toxicity Criteria.

Table 4. Genotype and haplotype distribution in our sample

Genotype		Patients *n* (%)	*MTHFR* 677					
			CC		CT		TT	
			143	(49.3)	119	(41.0)	28	(9.7)
MTHFR 1298	AA	128 (44.1)	41	(14.1)	59	(20.3)	28	(9.7)
	AC	132 (45.5)	73	(25.2)	59	(20.3)	–	–
	CC	30 (10.3)	29	(10.0)	1	(0.3)	–	–

The associations between the other clinical variables and OS and RFS in our study have been documented before, implying that our population is representative of patients undergoing 5-FU-based adjuvant chemotherapy [30].

We carried out SNP testing on archived tumor tissue. This could mean that we were unable to correct for the effects of loss of heterozygosity (LOH), which would produce fewer heterozygous individuals than expected. The fact that patient population genotype distribution was in HW equilibrium with genotype frequencies in accordance with previous reports [31] suggests that LOH is of minor importance in our patient population. Being a retrospective study there are limitations. First because of the nature of the disease we could not use a control group. Furthermore, additional tests like immunohistochemistry and MTHFR mRNA determination could not be carried out. Ideally, we would have liked to include more patients, but as demonstrated our study is one of the largest populations studied and treatment wise very homogenous making our results important in this field. Before applying our results, clinically larger studies must be conducted, so smaller survival differences (<10%–15%) can be detected and toxicity results can be confirmed.

An almost unique advantage of our population is that all patients were treated by identical regimes (5-FU/LV) presenting an exceptional opportunity for determining the effect of *MTHFR* genotypes on 5-FU/LV effect avoiding bias introduced by varying treatment regimes and effects of prior treatments. Monotherapy also means that other drugs do not blemish the analysis of genotype effects with non-related pharmacological mechanisms. At this time, a combination of oxaliplatin and 5-FU/LV is the standard treatment; in a reductionist model, knowing modifying factors for each drug is more informative than knowing modifying factors for the combination therapy alone. The former is only attainable by studying patients treated with a single agent.

Accumulating evidence suggests that *MTHFR* polymorphisms may not have functional effects in all tissues *in vivo*, that only the homozygous state predisposes to functional alternations and that polymorphisms only result in altered folate metabolism if the concentration of folate is low [32–35]. This is, perhaps, the explanation for the lack of clinical relevance for cancer treatment of *MTHFR* polymorphisms.

General concerns regarding previous studies of the association of these polymorphisms with treatment response include inclusion of pretreated patients making the results difficult to interpret, little statistical power, and inadequate statistical analysis, e.g. lacking multiple regression analysis or consideration of multiple testing. Overall, current clinical studies on the *MTHFR* polymorphisms have shown varying results with almost equal numbers showing no effect, a positive effect, or a negative effect on survival, response, or relapses.

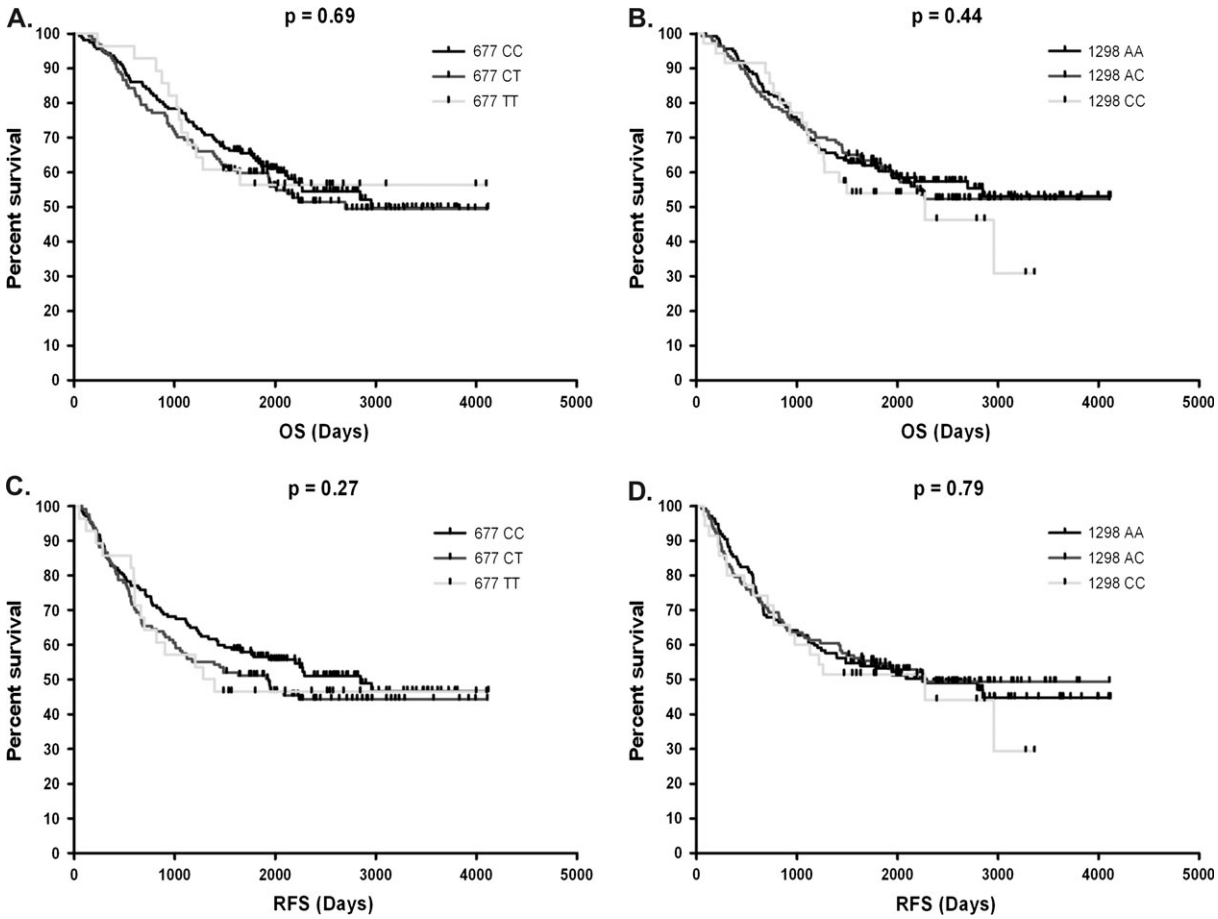

Figure 1. These Kaplan–Meier graphs show the relationship between the *MTHFR* 677C>T and *MTHFR* 1298A>C polymorphisms and overall survival (OS) and relapse-free survival (RFS). *P* values are indicated above curves for these three-way analyses. (A) OS: stratification according to *MTHFR* 677C>T. (B) OS: stratification according to *MTHFR* 1298A>C. (C) RFS: stratification according to *MTHFR* 677C>T. (D) RFS: stratification according to MTHFR 1298A>C.

Collectively, our study and previously published data indicate that the clinical significance of studying *MTHFR* polymorphisms alone is limited: genotyping restricted to these polymorphisms is inadequate to predict outcome in adjuvant 5-FU treatment after complete resection. A role in combination with other relevant SNPs, e.g. TYMS and others, cannot be excluded and has been suggested in other studies [17, 19].

Our study needs independent confirmation in a similar sized study, but further study of MTHFR polymorphisms should be conducted in combinatorial studies. Combinations of SNPs in a metabolic network are probably better predictors of response than single SNPs. However, combinatorial pharmacogentic studies require considerable sample sizes, well-defined target groups, and clinical relevance making them a daunting endeavor that nonetheless must be pursued. Such research could help to establish prognostic or predictive models that can be validated in large prospective studies.

From a theoretical point of view, there is no reason to think that susceptibility to 5-FU treatment is a monogenic trait since several proteins are involved in the pharmacokinetics and pharmacodynamics of 5-FU (and indeed all drugs). An approach, which combines several of the genes involved in 5-FU metabolism and mechanisms, will yield a better, more logical model for explaining individual variations in 5-FU efficacy.

In conclusion, the *MTHFR* 677C>T alone may have a weak effect on toxicity but neither polymorphism was predictive of 5-FU efficacy in the adjuvant setting.

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references

1. Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. CA Cancer J Clin 2005; 55(2): 74–108.
2. Cheradame S, Etienne MC, Chazal M et al. Relevance of tumoral folylpolyglutamate synthetase and reduced folates for optimal 5-fluorouracil efficacy: experimental data. Eur J Cancer 1997; 33(6): 950–959.
3. Danenberg PV, Danenberg KD. Effect of 5, 10-methylenetetrahydrofolate on the dissociation of 5-fluoro-2′-deoxyuridylate from thymidylate synthetase: evidence for an ordered mechanism. Biochemistry 1978; 17(19): 4018–4024.
4. Houghton JA, Maroda SJ Jr, Phillips JO, Houghton PJ. Biochemical determinants of responsiveness to 5-fluorouracil and its derivatives in xenografts of human colorectal adenocarcinomas in mice. Cancer Res 1981; 41(1): 144–149.
5. Keyomarsi K, Moran RG. Mechanism of the cytotoxic synergism of fluoropyrimidines and folinic acid in mouse leukemic cells. J Biol Chem 1988; 263(28): 14402–14409.

6. Canman CE, Tang HY, Normolle DP et al. Variations in patterns of DNA damage induced in human colorectal tumor cells by 5-fluorodeoxyuridine: implications for mechanisms of resistance and cytotoxicity. Proc Natl Acad Sci U S A 1992; 89(21): 10474–10478.
7. Yoshioka A, Tanaka S, Hiraoka O et al. Deoxyribonucleoside triphosphate imbalance. 5-Fluorodeoxyuridine-induced DNA double strand breaks in mouse FM3A cells and the mechanism of cell death. J Biol Chem 1987; 262(17): 8235–8241.
8. Buyse M, Zeleniuch-Jacquotte A, Chalmers TC. Adjuvant therapy of colorectal cancer. Why we still don't know. JAMA 1988; 259(24): 3571–3578.
9. Modulation of fluorouracil by leucovorin in patients with advanced colorectal cancer: evidence in terms of response rate. Advanced Colorectal Cancer Meta-Analysis Project. J Clin Oncol 1992; 10(6): 896–903.
10. Efficacy of adjuvant fluorouracil and folinic acid in colon cancer. International Multicentre Pooled Analysis of Colon Cancer Trials (IMPACT) investigators. Lancet 1995; 345(8955): 939–944.
11. Thirion P, Michiels S, Pignon JP et al. Modulation of fluorouracil by leucovorin in patients with advanced colorectal cancer: an updated meta-analysis. J Clin Oncol 2004; 22(18): 3766–3775.
12. Frosst P, Blom HJ, Milos R et al. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. Nat Genet 1995; 10(1): 111–113.
13. van der Put NM, Gabreels F, Stevens EM et al. A second common mutation in the methylenetetrahydrofolate reductase gene: an additional risk factor for neural-tube defects? Am J Hum Genet 1998; 62(5): 1044–1051.
14. Weisberg I, Tran P, Christensen B et al. A second genetic polymorphism in methylenetetrahydrofolate reductase (MTHFR) associated with decreased enzyme activity. Mol Genet Metab 1998; 64(3): 169–172.
15. Etienne MC, Ilc K, Formento JL et al. Thymidylate synthase and methylenetetrahydrofolate reductase gene polymorphisms: relationships with 5-fluorouracil sensitivity. Br J Cancer 2004; 90(2): 526–534.
16. Sohn KJ, Croxford R, Yates Z et al. Effect of the methylenetetrahydrofolate reductase C677T polymorphism on chemosensitivity of colon and breast cancer cells to 5-fluorouracil and methotrexate. J Natl Cancer Inst 2004; 96(2): 134–144.
17. Capitain O, Boisdron-Celle M, Poirier AL et al. The influence of fluorouracil outcome parameters on tolerance and efficacy in patients with advanced colorectal cancer. Pharmacogenomics J 2008; 8(4): 256–267.
18. Cohen V, Panet-Raymond V, Sabbaghian N et al. Methylenetetrahydrofolate reductase polymorphism in advanced colorectal cancer: a novel genomic predictor of clinical response to fluoropyrimidine-based chemotherapy. Clin Cancer Res 2003; 9(5): 1611–1615.
19. Etienne MC, Formento JL, Chazal M et al. Methylenetetrahydrofolate reductase gene polymorphisms and response to fluorouracil-based treatment in advanced colorectal cancer patients. Pharmacogenetics 2004; 14(12): 785–792.
20. Jakobsen A, Nielsen JN, Gyldenkerne N, Lindeberg J. Thymidylate synthase and methylenetetrahydrofolate reductase gene polymorphism in normal tissue as predictors of fluorouracil sensitivity. J Clin Oncol 2005; 23(7): 1365–1369.
21. Lurje G, Zhang W, Yang D et al. Thymidylate synthase haplotype is associated with tumor recurrence in stage II and stage III colon cancer. Pharmacogenet Genomics 2008; 18(2): 161–168.
22. Marcuello E, Altes A, Menoyo A et al. Methylenetetrahydrofolate reductase gene polymorphisms: genomic predictors of clinical response to fluoropyrimidine-based chemotherapy? Cancer Chemother Pharmacol 2006; 57(6): 835–840.
23. Ruzzo A, Graziano F, Loupakis F et al. Pharmacogenetic profiling in patients with advanced colorectal cancer treated with first-line FOLFOX-4 chemotherapy. J Clin Oncol 2007; 25(10): 1247–1254.
24. Ruzzo A, Graziano F, Loupakis F et al. Pharmacogenetic profiling in patients with advanced colorectal cancer treated with first-line FOLFIRI chemotherapy. Pharmacogenomics J 2008; 8(4): 278–288.
25. Suh KW, Kim JH, Kim DY et al. Which gene is a dominant predictor of response during FOLFOX chemotherapy for the treatment of metastatic colorectal cancer, the MTHFR or XRCC1 gene? Ann Surg Oncol 2006; 13(11): 1379–1385.
26. Terrazzino S, Agostini M, Pucciarelli S et al. A haplotype of the methylenetetrahydrofolate reductase gene predicts poor tumor response in rectal cancer patients receiving preoperative chemoradiation. Pharmacogenet Genomics 2006; 16(11): 817–824.
27. Wisotzkey JD, Toman J, Bell T et al. MTHFR (C677T) polymorphisms and stage III colon cancer: response to therapy. Mol Diagn 1999; 4(2): 95–99.
28. Zhang W, Press OA, Haiman CA et al. Association of methylenetetrahydrofolate reductase gene polymorphisms and sex-specific survival in patients with metastatic colon cancer. J Clin Oncol 2007; 25(24): 3726–3731.
29. Schwab M, Zanger UM, Marx C et al. German 5-FU Toxicity Study Group. Role of genetic and nongenetic factors for fluorouracil treatment-related severe toxicity: a prospective clinical trial by the German 5-FU Toxicity Study Group. J Clin Oncol 2008; 26(13): 2131–2138.
30. The Meta-Analysis Group In Cancer. Toxicity of fluorouracil in patients with advanced colorectal cancer: effect of administration schedule and prognostic factors. J Clin Oncol 1998; 16(11): 3537–3541.
31. Shi M, Caprau D, Romitti P et al. Genotype frequencies and linkage disequilibrium in the CEPH human diversity panel for variants in folate pathway genes MTHFR, MTHFD, MTRR, RFC1, and GCP2. Birth Defects Res A Clin Mol Teratol 2003; 67(8): 545–549.
32. Jensen SA, Vainer B, Sørensen JB. The prognostic significance of thymidylate synthase and dihydropyrimidine dehydrogenase in colorectal cancer of 303 patients adjuvantly treated with 5-fluorouracil. Int J Cancer 2006; 120(3): 694–701.
33. Huang Y, Han S, Li Y et al. Different roles of MTHFR C677T and A1298C polymorphisms in colorectal adenoma and colorectal cancer: a meta-analysis. J Hum Genet 2007; 52(1): 73–85.
34. Friso S, Choi SW, Girelli D et al. A common mutation in the 5,10-methylenetetrahydrofolate reductase gene affects genomic DNA methylation through an interaction with folate status. Proc Natl Acad Sci U S A 2002; 99(8): 5606–5611.
35. Jacques PF, Bostom AG, Williams RR et al. Relation between folate status, a common mutation in methylenetetrahydrofolate reductase, and plasma homocysteine concentrations. Circulation 1996; 93(1): 7–9.
36. Schwartz SM, Siscovick DS, Malinow MR et al. Myocardial infarction in young women in relation to plasma total homocysteine, folate, and a common variant in the methylenetetrahydrofolate reductase gene. Circulation 1997; 96(2): 412–417.
37. Yamada K, Chen Z, Rozen R, Matthews RG. Effects of common polymorphisms on the properties of recombinant human methylenetetrahydrofolate reductase. Proc Natl Acad Sci U S A 2001; 98(26): 14853–14858.