Thymidylate Synthase and Methylenetetrahydrofolate Reductase Gene Polymorphisms and Toxicity to Capecitabine in Advanced Colorectal Cancer Patients

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Abstract

Purpose: To evaluate the effect of thymidylate synthase (*TYMS*) and methylenetetrahydrofolate reductase (*MTHFR*) genotypes on toxicity in patients treated with capecitabine for advanced colorectal cancer and to determine the effect of these polymorphisms on the pretreatment levels of serum folate and plasma homocysteine.

Experimental Design: Fifty-four patients with a diagnosis of metastatic colorectal cancer were treated with fixed-dose capecitabine. Germ line DNA from patients was genotyped for *TYMS* TSER, TSER*3G>C, and 3′-untranslated 6 bp insertion/deletion (3′ untranslated region insertion/deletion), and *MTHFR* c.677C>T and c.1298A>C using PCRs and RFLP. Toxicity was graded by National Cancer Institute Common Toxicity Criteria version 2.0. Response was assessed by Response Evaluation Criteria in Solid Tumors.

Results: *MTHFR* c.677C>T and c.1298A>C genotypes and diplotypes predicted for grade 2/3 toxicities, whereas the *TYMS* genotypes had no influence. *MTHFR* c.677 genotype tended to predict overall survival ($P = 0.08$). *MTHFR* c.677 influenced pretreatment homocysteine ($P < 0.05$) and serum folate levels ($P < 0.05$). Multivariate analysis suggests that MTHFR c.1298 is an independent predictor of toxicity.

Conclusions: This study suggests that common genetic variation in *MTHFR* but not *TYMS* may be useful for predicting toxicity from capecitabine in patients with advanced colorectal cancer. In addition, *MTHFR* single nucleotide polymorphisms predicted serum folate and plasma homocysteine levels, and, combined, these factors may be important predictors of capecitabine-induced toxicity.

Despite recent advances in the treatment of metastatic colorectal cancer, 5-fluorouracil (5-FU) remains the mainstay of therapy (1). Capecitabine is a rationally designed oral fluoropyrimidine that is being increasingly used both in the adjuvant and metastatic setting. Capecitabine preferentially delivers 5-FU to the tumor via a three-step enzymatic conversion, the final step being catalyzed by thymidine phosphorylase, which has a higher activity within tumor compared with healthy tissue (2).

Little is known about predictors of toxicity, response, and survival in patients with colorectal cancer treated with capecitabine. It is widely recognized that apart from confounding environmental and physiologic factors, inherited variability in drug-metabolizing enzymes, drug transporters, and drug targets plays an important role in the variability of treatment outcomes, when drugs are prescribed uniformly to all patients. Drug-related toxicity relates almost exclusively to nontumor tissue and, therefore, inherited polymorphisms can play a central role in determining toxicity. Pharmacogenetic screening may identify those patients that are likely to respond and those that are likely to have severe toxicity due to cytotoxic chemotherapy. As toxicity remains the major limitation to adequate dosing, the ability to predict toxicity before the administration of chemotherapy, and to provide individualized treatment, would likely result in improved outcomes.

Thymidylate synthase (TS) is a key enzyme in nucleotide biosynthesis, and is the main intracellular target of the active metabolite of 5-FU, fluorodeoxyuridylate, which forms a ternary complex with TS and 5,10-methylenetetrahydrofolate (5,10-MTHF). This complex prevents methylation of dUMP to

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dTMP, the sole *de novo* intracellular source of thymidylate. It is well established that overexpression of both TS mRNA and protein levels in colorectal cancer confers a poor response to 5-FU (3–6). The wide interpatient variation in TS levels can be ascribed in part to polymorphisms in the promoter and 3′-untranslated regions (3′UTR) of the TS gene (*TYMS*).

The promoter enhancer region of *TYMS* (TSER) contains a polymorphic 28-bp tandem repeat sequence. The most common alleles have two (TSER*2 allele) or three tandem repeats (TSER*3). *In vitro* expression studies have shown a positive relationship between the number of tandem repeats and the transcriptional activity of *TYMS*, with TSER*3 having 2.6-fold greater activity than TSER*2 (7). Moreover, homozygosity for TSER*3 is associated with lower plasma folate concentrations (8). An upstream stimulatory factor E-box consensus element was identified within the tandem repeat of the TSER*2 and TSER*3 alleles (9, 10). *In vitro* experiments showed that the nuclear factors USF-1 and USF-2 bind to these consensus sites and transactivate *TYMS*. In addition, a common G-to-C transversion in the second of the three 28-bp repeats of TSER*3 has been identified. This single nucleotide polymorphism (SNP) changes a critical residue in the upstream stimulatory factor E-box consensus element, which leads to a decrease in *TYMS* transcription such that TSER*3C has lower activity than TSER*3G, but similar activity to TSER*2.

A second common polymorphism has been reported at position 1494 in the 3′UTR of *TYMS* (11). 3′UTRs modulate gene regulation at a posttranscriptional level through control of mRNA stability. A 6-bp insertion/deletion (indel) polymorphism in *TYMS* 3′UTR is associated with decreased *TYMS* mRNA stability *in vitro* and reduced expression of TS protein in colorectal tumor tissue (12). In colorectal cancer patients treated with adjuvant 5-FU, the indel was shown to have prognostic value in predicting survival (13).

Optimal inhibition of TS requires elevated cellular concentrations of 5,10-MTHF. MTHFR plays a central role in the metabolism of folate, catalyzing the reduction of 5,10-MTHF to 5-methyltetrahydrofolate, the primary circulatory form of folate and methyl donor for the remethylation of homocysteine to methionine. Two common nonsynonymous SNPs, c.677C>T ($Ala^{222}Val$) and c.1298A>C ($Glu^{429}Ala$), with reduced enzymatic conversion of 5,10-MTHF to 5-methyltetrahydrofolate, have been described in the MTHFR gene (*MTHFR*; refs. 14–16). Because the MTHFR variant allozymes have reduced enzymatic activity compared with wild-type enzyme *in vitro*, it is anticipated that patients with variant alleles will have elevated cellular 5,10-MTHF and, as a consequence, increased sensitivity to fluoropyrimidines such that they have an improved response to fluoropyrimidine-based therapies and possibly an elevated risk of toxicity compared with patients with the wild-type or common alleles. There is clinical evidence to support this hypothesis (14, 16–19).

TYMS polymorphisms and *MTHFR* c.677C>T are reported to be determinants of plasma homocysteine and serum and RBC folate concentrations (8, 20, 21). The influence of pretreatment folate and homocysteine concentrations on treatment outcomes to TS inhibitors and folate antagonists is increasingly being recognized (22, 23). Folate and homocysteine levels seem to be correlated with the extent of TS inhibition and the retention of the TS-FdUMP complex (24). Previous murine studies have indicated that folate levels may predict for the cytotoxic efficacy of 5-FU (25). In addition, we have previously shown that pretreatment levels of serum folate can predict for worse toxicity during the first treatment cycle with capecitabine (26). No studies to date have examined the influence of polymorphisms in *TYMS* and *MTHFR* on homocysteine and folate on clinical outcomes in patients with advanced colorectal cancer.

We undertook a prospective pharmacogenetic study in a cohort of advanced colorectal cancer patients treated with capecitabine. The aim of the study was to determine whether germ line polymorphisms in *TYMS* and *MTHFR* predict toxicity. We also report mature survival and response data. Furthermore, we investigated the ability of *TYMS* and *MTHFR* polymorphisms and pretreatment levels of folate and homocysteine to predict toxicity from capecitabine treatment.

Materials and Methods

Patients. The study was conducted at three different hospitals in New South Wales, Australia, each of which followed the same protocol, which was coordinated from a single center. Patients were eligible for entry into the study if they had locally advanced or metastatic colorectal cancer with measurable or evaluable disease. Patients could have previously received up to two prior chemotherapy regimens for metastatic disease. If administered, adjuvant therapy should have been completed ≥6 months before study entry. Histologic or cytologic confirmation of colorectal adenocarcinoma was required. Patients had to be at least 18 years old and have a life expectancy >12 weeks. Eastern Cooperative Oncology Group performance status was required to be between 0 and 2. Patients were not included if they had active or extensive brain metastases, active systemic infection, inflammatory bowel disease, unstable cardiac disease, or untreated vitamin B12 deficiency. Patients who were pregnant or actively lactating were excluded. Patients had to be able to be changed from warfarin to enoxaparin sodium (27). Patients were not enrolled if initial evaluations revealed significant abnormalities in neutrophils ($<1.5 \times 10^9$/L); platelets ($<100 \times 10^9$/L); serum creatinine or serum bilirubin (>1.5× upper limit of normal); and alanine aminotransferase, aspartate aminotransferase, or alkaline phosphatase (>3× upper limit of normal). All patients were treated with capecitabine at a dose of 2,000 mg twice daily on days 1 to 14, every 3 weeks. The study received prior local ethical board approval and was conducted according to the Declaration of Helsinki and its subsequent amendments. All patients gave written informed consent before participation in the trial. Efficacy of fixed-dose capecitabine and preliminary data on the relationships between plasma deoxynucleoside concentrations and *TYMS* TSER genotype and folate status with capecitabine treatment outcomes have been published elsewhere (26, 28).

Evaluation of patients. Complete history was recorded, full physical examination was done, and blood samples were collected at baseline. Baseline blood analyses included full blood count, biochemistry, liver function tests, vitamin B12, carcinoembryonic antigen, serum and red cell folate, and plasma homocysteine. Serum and red cell folate and plasma homocysteine were measured in a central laboratory using National Committee for Clinical Laboratory Standards procedures. Serum and red cell folate were measured using a competitive binding receptor assay (Access Immunoassay, Beckman and Coulter, Inc.). Plasma homocysteine was measured using an immunoassay (Abbott Axsym Analyser, Abbott). Samples were not batched but analyzed as patients were enrolled. Blood samples were obtained for DNA isolation and determination of genotypes. Baseline computed tomography imaging of the chest, abdomen, and pelvis were obtained within 3 weeks of treatment commencement. Patients were reviewed weekly during cycle 1 and then every 3 weeks for safety assessment. All safety evaluations were graded according to the National Cancer Institute

Table 1. Demographic and clinical characteristics (n = 54)

Characteristics	No. patients (%)
Male	35 (65%)
Age (y), median (range)	72 (42-86)
Body weight (kg), median (range)	71 (44.3-117.8)
Performance status	
0	18 (33%)
1	33 (61%)
2	3 (6%)
Primary tumor site	
Colon	38 (70%)
Rectal	15 (28%)
Unknown	1 (2%)
No. metastatic sites	
1	32 (58%)
2	13 (24%)
≥3	9 (15%)
Prior adjuvant chemotherapy (5-FU)	13 (24%)
Prior chemotherapy for metastatic disease	2 (4%)
Oxaliplatin/5-FU	1 (2%)
Irinotecan/5-FU	1 (2%)
Prior pelvic radiotherapy	6 (11%)

Common Toxicity Criteria version 2.0. Hand-foot syndrome was classified as grade 1 (numbness, dysesthesia, painless swelling, or erythema not disrupting normal activities), grade 2 (painful erythema with swelling or affecting daily living activities), or grade 3 (moist desquamation, ulceration, blistering, severe pain, or any symptoms leading to an inability to work or to perform daily living activities; ref. 29). Tumor response was assessed after every two cycles with repeat computed tomography of chest, abdomen, and pelvis. Tumor response or progression was recorded using the Response Evaluation Criteria in Solid Tumors (30).

Genotype analysis. Genomic DNA was isolated from whole blood using DNeasy tissue kit (Qiagen). *TYMS* TSER genotypes were determined using PCR followed by gel electrophoresis, as previously described (31). For TSER*3G>C SNP assignment, PCR amplicons were digested with *Hae*III and the digested bands were separated by gel electrophoresis using low-melt agarose (10). Genotypes were scored based on the following expected allelic fragment patterns: TSER*2, 12, 44, 47, and 66 bp; TSER*3G, 12, 28, 44, 47, and 66 bp; and TSER*3C, 12, 44, 47, and 94 bp. For the *TYMS* 3′-UTR indel, DNA was amplified using the primers of Ulrich et al. (11). Amplicons were digested with *Dra*I and genotypes were assigned based on the following gel patterns: +6 bp/+6 bp; 88, 70 bp, +6 bp/-6 bp; 152, 88, 70 bp, -6 bp/-6 bp; 152 bp. *MTHFR* c.677C>T genotypes were determined using PCR followed by gel electrophoresis, as previously described (14). PCR amplicons were digested with *Hin*fI and the digested bands were separated by gel electrophoresis using high-resolution agarose. Genotypes were scored based on the following gel patterns: C/C, 198 bp; C/T, 198, 175, 23 bp; T/T, 175, 23 bp. For *MTHFR* c.1298A>C, DNA was amplified using the primers of van der Put et al. (15). The 163-bp amplicon was digested with *Mbo*II and genotypes were scored based on the following gel patterns: A/A, 56, 28, 31, 30, 18 bp; A/C, 84, 56, 28, 31, 30, 18 bp; and C/C, 84, 31, 30, 18 bp.

Statistics. Pairwise linkage disequilibrium between *MTHFR* c.677 and c.1298 alleles was estimated by a log-linear model and the extent of disequilibrium was expressed in terms of D', which is the ratio of the unstandardized coefficient to its maximal/minimal value. *MTHFR* haplotypes were estimated using Polymorphism and Haplotype Analysis Suite version 0.9 (32). For statistical analysis, overall toxicity was dichotomized as either mild (grades 0 and 1) or moderate to severe (grades 2 and 3) a priori. Subgroup analysis was then done on each adverse event individually and combined. The χ^2 and Fisher's exact tests were used to compare the rate of toxicities and response between *TYMS* and *MTHFR* genotypes and *MTHFR* diplotypes. Treatment outcomes (toxicities and clinical response) were compared between TSER*3/TSER*3, TSER*3/TSER*2, and TSER*2/TSER*2 patients. Because TSER*3G has a higher transcriptional activity than TSER*3C or TSER*2 (9, 10), outcomes were also compared between patients with two TSER*3G alleles (TSER*3G/TSER*3G), one TSER*3G allele (TSER*3G/TSER*3C, TSER*3G/TSER*2), and no TSER*3G alleles (TSER*3C/TSER*3C, TSER*3C/TSER*2, TSER*2/TSER*2).

t tests were used to compare analyte levels between *TYMS* and *MTHFR* genotypes, *MTHFR* diplotypes, and between patients with grade 0/1 and grade 2/3 toxicities. Measurements were missing for homocysteine (n = 15), serum folate (n = 6), red cell folate (n = 7), and vitamin B12 (n = 5). All missing data were imputed using a multiple imputation method before running the analyses. Running the iterative imputation algorithm for 50 iterations, 10 sets of imputed values for the missing data points were generated (33).

Log-rank tests and Cox regression analyses were used to assess univariate relationships between genotypes, analyte levels, and overall survival. Multiple logistic regression was used to identify independent predictors of grade 2/3 toxicity. Factors selected for inclusion on multivariate analysis had to be significant at $P < 0.05$ on univariate analysis. The odds ratio (OR) and its 95% confidence interval (95% CI) were used to quantify the extent of any association. Two-tailed tests with a significance level of 5% were used throughout. Analysis was done using SPSS version 11.5 (SPSS, Inc.) and the R package MICE for multiple imputation of missing data (34).

Results

Patients and treatment outcome. A total of 56 patients with metastatic or locally advanced colorectal cancer were enrolled from three centers in Australia between January 2002 and August 2003. Blood was not collected from two patients; therefore, genotype-outcome analyses were conducted on 54 patients. The demographic and clinical characteristics of the patients are summarized in Table 1.

Five patients were still receiving capecitabine at the time of analysis. The median duration of therapy with capecitabine was 107 days (range 21-232 days) and the median number of cycles received was 4.9 (range 1-8 cycles). The most common treatment-related adverse events are reported in Table 2. Overall, capecitabine was well tolerated with no grade 4 nonhematologic or grade 3/4 hematologic adverse events recorded. Diarrhea, oral stomatitis, and fatigue were the predominant adverse events reported. Adverse events led to the cessation of treatment in eight patients (15%) and of these, five patients ceased treatment after cycle 1. The most

Table 2. Grade 2 and 3 (%) adverse events experienced by patients over the entire treatment period (n = 54)

Toxicity	Patients (%)	
	Grade 2	Grade 3
Fatigue	28 (15)	2 (1)
Hand-foot syndrome	11 (6)	9 (5)
Stomatitis	10 (19)	0
Diarrhea	22 (12)	7 (4)
Nausea and vomiting	9 (5)	0
Anemia	13 (7)	0
Liver toxicity	4 (2)	0

Table 3. Incidence of toxicity by genotype

Genotype	Patients (%)	
	Grade 0/1	Grade 2/3
TSER		
3RG/3RG	4 (50)	4 (50)
3RG/3C and 3RG/2R	10 (46)	12 (55)
Absence of 3RG	10 (44)	13 (57)
3′UTR indel		
6 bp/+6 bp	9 (47)	10 (53)
+6 bp/-6 bp	15 (52)	14 (48)
-6 bp/-6 bp	1 (17)	5 (83)
MTHFR 677		
CC	11 (41)	16 (59)
CT	8 (40)	12 (60)
TT	6 (86)	1 (14)
MTHFR 1298		
AA	17 (68)	8 (32)
AC	4 (17)	19 (83)
CC	4 (67)	2 (33)

frequent adverse event leading to discontinuation was diarrhea. There were no adverse event–related deaths during the study.

TYMS *polymorphisms and toxicity*. The distributions of TSER genotypes and of the SNP G>C at the 12th nucleotide in the second repeat of the TSER*3 allele were as follows: 8 patients were TSER*3G/TSER*3G, 8 patients were TSER*3G/TSER*3C, 14 patients were TSER*3G/TSER*2, and 23 patients had no TSER*3G allele (6 patients TSER*2/TSER*2, 15 patients TSER*2/TSER*3C, 2 patients TSER*3C/TSER*3C). One patient had a TSER*2/TSER*4 genotype and was not included in analysis. The observed genotypes were in agreement with those predicted by the Hardy-Weinberg equilibrium. The allelic frequencies of TSER*3G, TSER*3C, TSER*2, and TSER*4 were 35%, 25%, 39%, and 1%, respectively. For the *TYMS* 3′UTR indel, 19, 29, and 6 patients had +6 bp/+6 bp, +6 bp/-6 bp, and -6 bp/-6 bp genotypes, respectively. The distribution of the genotypes was in agreement with the Hardy-Weinberg equilibrium. The frequency of the -6 bp allele was 38%. No relationships between *TYMS* TSER, TSER*3G>C, and 3′UTR genotypes, and overall toxicity were observed on univariate Fisher's exact tests (Table 3).

MTHFR *polymorphisms and haplotypes, and toxicity*. Twenty-seven patients had the *MTHFR* c.677 C/C genotype, twenty patients had the C/T genotype, and seven patients had the T/T genotype. For c.1298A>C, 25, 23, and 6 patients had the A/A, A/C, and C/C genotypes, respectively. Genotype frequencies were in agreement with the Hardy-Weinberg equilibrium. The frequencies of c.677 T and c.1298 C alleles were 31% and 32%, respectively.

Consistent with previous findings, *MTHFR* c.677C>T and c.1298A>C were in complete linkage disequilibrium ($D' = 1$, $P < 0.05$; refs. 35, 36). Three haplotype identities C-A (c.677C>T-c.1298A>C), C-C, and T-A were observed. The frequencies of the haplotypes were 35%, 33%, and 32%, respectively.

On analysis patients with the *MTHFR* c.677 T/T genotype had a lower incidence of grade 2/3 toxicity than patients with C/T and C/C genotypes (OR, 0.1; 95% CI, 0.01-1.0; $P < 0.05$; Fig. 1A). On further analysis, patients with C/T and T/T genotypes experienced less grade 2/3 fatigue (4%, 1 of 27) compared with patients with the C/C genotype (33%, 9 of 29; OR, 0.08; 95% CI, 0.009-0.66; $P < 0.05$). Conversely, C/T and T/T patients seemed to experience a higher incidence of grade 2/3 hand-foot syndrome (7%, 7 of 27 patients) than patients with the C/C genotype (4%, 1 of 27 patients; OR, 9.1; 95% CI, 1.03-80.09; $P = 0.05$).

A significant association was also observed between the c.1298A>C genotype and overall combined toxicities on univariate analysis (Fig. 1B). Patients with the A/A genotype suffered less grade 2/3 toxicity (8 of 25 patients, 32%) than patients with A/C and C/C genotypes (21 of 29 patients, 72%; OR, 5.6; 95% CI, 1.73-17.98; $P < 0.01$). On further analysis, A/A patients experienced less grade 2/3 fatigue (4%, 1 of 25) compared with patients with A/C and C/C genotypes (31%, 9 of 29; OR, 10.8; 95% CI, 1.26-92.67; $P < 0.05$).

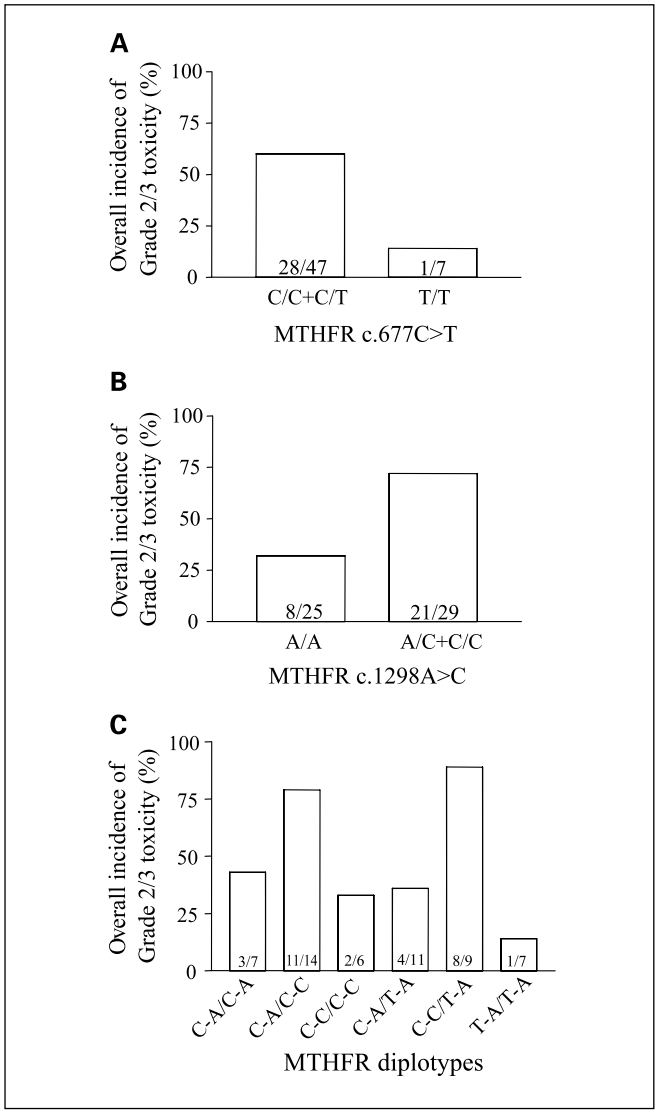

Fig. 1. Incidence of grade 2/3 toxicity experienced over the entire treatment period by patients treated with capecitabine by MTHFR genotype. *A, MTHFR* c.677C>T genotypes ($n = 54$, $P < 0.05$, Fisher's exact test). *B, MTHFR* c.1298A>C genotypes ($n = 54$, $P < 0.01$, Fisher's exact test). *C, MTHFR* diplotypes (c.677C>T-c.1298A>C; $n = 54$, $P < 0.05$, χ^2 test).

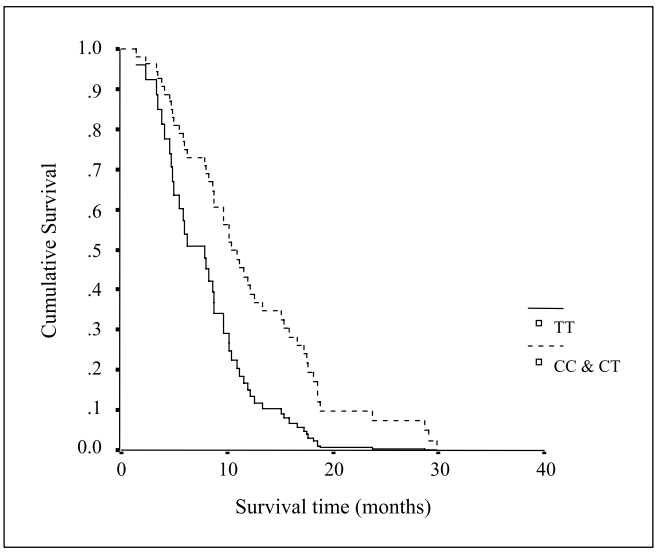

Fig. 2. Kaplan-Meier survival curves showing cumulative survival of capecitabine-treated patients by *MTHFR* c.677C>T genotype (T/T vs C/T+C/C; $P = 0.08$, $n = 54$).

Consistent with the *MTHFR* SNP data, the incidence of grade 2/3 toxicity was related to *MTHFR* diplotype (Fig. 1C). Patients with the T-A/T-A (c.677C>T-c.1298A>C) diplotype experienced less grade 2/3 toxicity (1 of 7 patients, 14%) than patients with other diplotypes (28 of 47 patients, 60%; OR, 0.09; 95% CI, 0.01-0.81; $P < 0.05$). In addition, the majority of the patients with one or two C-C haplotypes experienced grade 2/3 toxicity (21 of 29 patients, 72%) whereas considerably less patients with no C-C haplotypes experienced grade 2/3 toxicity (8 of 25 patients, 32%; OR, 6.8; 95% CI, 2.05-22.27; $P < 0.01$).

Efficacy of palliative capecitabine and effect of polymorphisms. At the time of analysis, 49 of the 56 patients had died. The cause of death in all patients was disease progression. Two patients were not evaluable, as they did not have radiological follow-up. The overall response rate in evaluable patients was 25% (13 of 52 patients). Eighteen patients had stable disease and the remaining 21 patients (40%) had disease progression. Median survival was 10.2 months (95% CI for median survival, 8.4-11.9) and median time to disease progression was 7.9 months (95% CI for time to disease progression, 6.1-9.8). No significant relationships between *TYMS* polymorphisms and clinical response or survival were observed. No significant relationships between *MTHFR* SNPs, diplotype, and clinical response were observed on univariate analyses. Patients with the *MTHFR* c.677 T/T genotype tended to have a shorter overall survival than patients with C/T and C/C genotypes (hazard ratio, 0.5; 95% CI, 0.2-1.1; $P = 0.08$; Fig. 2). No significant associations between *MTHFR* c.1298 genotypes or *MTHFR* diplotypes and survival were observed.

Pretreatment analyte levels. Median pretreatment plasma homocysteine was 13.5 μmol/L (range, 8-47 μmol/L; $n = 39$), serum folate was 16.3 nmol/L (range, 6-45 nmol/L; $n = 48$), red cell folate was 613.5 nmol/L (range, 358-1,774 nmol/L; $n = 37$), and vitamin B12 was 265 pmol/L (range, 17-945 pmol/L; $n = 49$). The relationships between analyte levels and toxicity were assessed. No associations were observed between serum and red cell folate, homocysteine or vitamin B12, and overall toxicity, and there were no relationships between the analyte levels and overall survival after using multiple imputations to impute the missing data.

The relationships between *TYMS* and *MTFHR* genotypes and homocysteine, red cell and serum folate and vitamin B12 were assessed after using multiple imputations to impute the missing data. *TYMS* polymorphisms were not associated with analyte levels. Patients with the *MTHFR* c.677 T/T genotype had significantly lower levels of pretreatment serum folate than patients with C/T and C/C genotypes ($P < 0.05$; Fig. 3A). Furthermore, T/T patients had higher pretreatment plasma homocysteine than other patients ($P < 0.05$; Fig. 3B) and

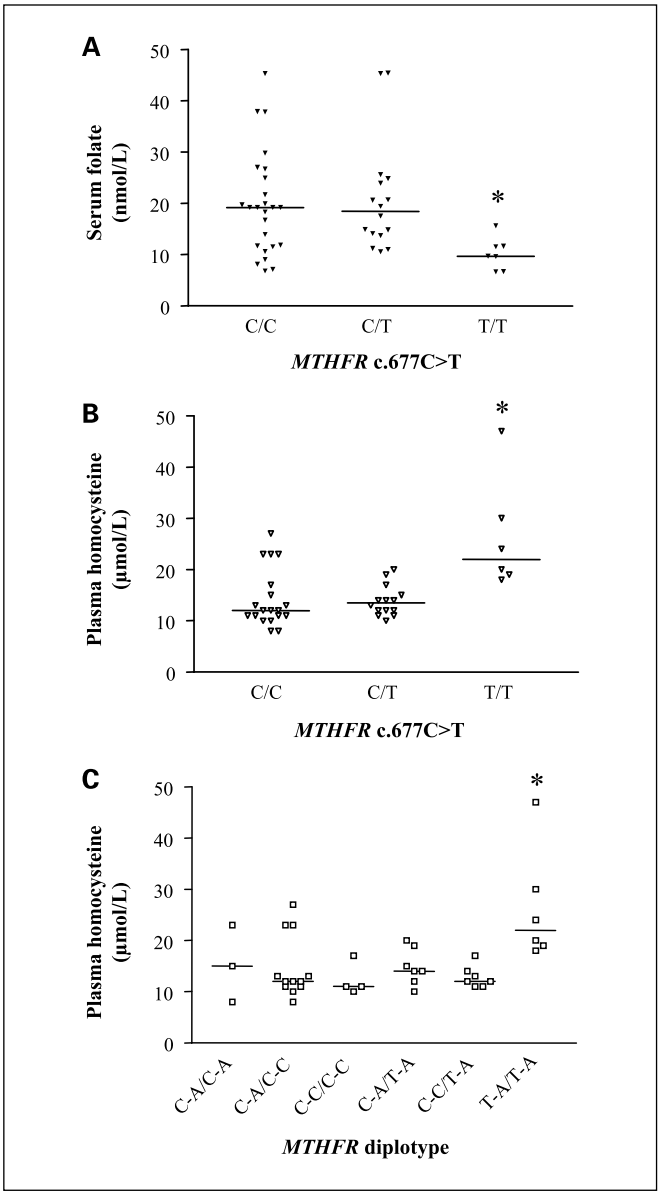

Fig. 3. Relationships of *MTHFR* genotypes and diplotypes, with serum folate and plasma homocysteine. Relationships between *MTHFR* c.677C>T and (*A*) serum folate (T/T vs C/C+C/T; $P < 0.05$, $n = 48$) and (*B*) plasma homocysteine (T/T vs C/C+C/T; $P < 0.05$, $n = 39$). Relationships between (*C*) *MTHFR* c.1298A>C and plasma homocysteine (A/A vs A/C+C/C; $P < 0.05$, $n = 39$) and (*D*) *MTHFR* diplotypes (c.677C>T-c.1298A>C) T-A/T-A vs other diplotypes with plasma homocysteine ($P < 0.05$, $n = 39$). *, $P \leq 0.05$.

tended to have lower levels of vitamin B12 ($P = 0.07$). Patients with the c.1298 A/A genotype tended to have higher plasma homocysteine ($P = 0.06$) and vitamin B12 ($P = 0.06$) than individuals with A/C and C/C genotypes. *MTHFR* genotypes were not related to red cell folate levels.

On analysis of *MTHFR* diplotype, T-A/T-A patients had significantly higher levels of plasma homocysteine ($P < 0.05$; Fig. 3C) and lower levels of serum folate ($P < 0.05$, data not shown) compared with other diplotypes. A trend was observed between those patients with C-C haplotype and raised levels of plasma homocysteine ($P = 0.08$). No associations were observed between diplotype and the other analyte levels studied.

Multivariate analysis of predictors of toxicity. *MTHFR* c.677 and c.1298 genotypes and diplotypes were considered as possible predictors for toxicity on multiple logistic regression following univariate analysis. *MTHFR* c.1298 genotype was found to be an independent predictor of toxicity such that A/A patients experienced less toxicity than patients with the A/C and C/C genotypes (OR, 0.06; 95% CI, 0.01-0.3, $P < 0.05$).

Discussion

The goal of many pharmacogenetic investigations is to identify genetic markers that predict either drug toxicity or efficacy. In the case of cytotoxic agents, such predictive markers could be used to identify those cancer patients before drug administration that are unlikely to respond, and those at high risk of excessive toxicity. Such information would allow the individualization of chemotherapy regimens, thereby allowing a full treatment course to be administered while maintaining quality of life. To bring this concept into routine clinical practice, validated predictors of outcome are required that cause minimal inconvenience to the patient. The primary objective of the present study was to evaluate whether common germ line polymorphisms in *TYMS* and *MTHFR* predict toxicity in patients with metastatic colorectal cancer receiving capecitabine. In addition, we sought to investigate whether *TYMS* and *MTHFR* polymorphisms are associated with folate status, and to investigate the combined effect of these factors in predicting toxicity from capecitabine. We also report mature survival data and the ability of these polymorphisms to predict for response and survival in this patient group.

We have shown that SNPs in *MTHFR* are predictive of toxicity to capecitabine. Moreover, a significant relationship was observed between *MTHFR* diplotypes and toxicity. To our knowledge, this is the first prospective study to investigate the relationship between *MTHFR* haplotypes and clinical outcomes to a fluoropyrimidine therapy in a palliative setting. Of interest is the finding that germ line *TYMS* polymorphisms did not predict for toxicity, clinical response, or survival to capecitabine chemotherapy in this cohort.

Whereas a number of studies have investigated the effect of TSER genotypes on toxicity, response, and overall survival in patients treated with 5-FU, the results are conflicting (17, 37–39). Two studies investigated the role of TSER genotype on toxicity and both showed an inverse relationship between the numbers of tandem repeats in TSER and the severity of toxicity experienced by patients (37, 40). This association, however, did not remain significant following the consideration of the G>C SNP in the TSER*3 allele, consistent with our findings (41).

The *TYMS* 3′UTR indel has not been as extensively studied as TSER, with only one clinical study investigating the association between the 3′UTR indel and toxicity from fluoropyrimidine-based therapies (40). No relationship was observed, which is consistent with our results. The prognostic value of *TYMS* 3′UTR indel in colorectal cancer has been investigated in only two studies, both reporting conflicting results (13, 41). The value, therefore, of *TYMS* polymorphisms in predicting outcome to 5-FU–based therapy is not clearly established and requires further investigation.

In our patient cohort, TSER genotypes did not influence clinical response or overall survival to capecitabine therapy. In a study of patients with advanced gastric cancer, Ruzzo and colleagues (42) showed that high-expression TSER genotypes predicted for poor overall survival from cisplatin and 5-FU–based therapy. Similarly, Kawakami and colleagues (43) found the presence of least one high *TS* expression genotype, TSER*3G/*3G, TSER*3G/*3C, TSER*2/*3G or 3′UTR +6 bp/+6 bp, predicted for worse overall survival in patients with gastric cancer receiving adjuvant fluoropyrimidine therapies compared with patients without any high expression genotypes. These findings contradict our results; however, they may be explained by differences in the study populations. Both Ruzzo and Kawakami studied patients with a history of gastric cancer who were treated with combination chemotherapy using differing fluoropyrimidine regimes, and in the article by Kawakami et al., this was given as the adjuvant therapy. Further differences may arise from the comparatively limited sample size enrolled in our study, which may have served to underestimate any effect of the *TYMS* genotype. However, both articles highlight the importance of assessing the G>C transversion in *TYMS* 5′UTR in combination with the 3′UTR indel in future *TYMS* association studies (44).

Given the interplay between TS and MTHFR in mediating 5-FU cytotoxicity, we investigated whether common *MTHFR* SNPs predict for toxicity in patients receiving capecitabine treatment (45–47). The *MTHFR* c.677 T/T genotype was predictive of less grade 2/3 toxicity compared with the other genotypes. The *MTHFR* c.1298 was also predictive of toxicity, with an A/A genotype being protective for grade 2/3 toxicity, particularly fatigue.

The observed relationship between *MTHFR* SNPs and toxicity are not in agreement with the results of two previous pharmacogenetic studies that used germ line DNA (17, 48). In a study of advanced breast cancer, *MTHFR* c.677 and c.1298 genotypes were not related to toxicity from capecitabine therapy (48). However, the incidence of overall toxicity in this study was low (15 of 105 patients), which may have limited the power of the study to detect any associations. In addition, it must be considered that Largillier and colleagues assessed the associations between genotypes and the incidence of grade 3/4 toxicity; this contrasts with our study where, given the low anticipated toxicity from capecitabine, we tested genotype associations with grade 2/3 toxicity. In another study of advanced colorectal cancer patients receiving fluoropyrimidine-based therapies, similar incidences of neutropenia, diarrhea, nausea, vomiting, and mucositis were observed for patients with differing c.677 genotypes, although only five patients with T/T genotypes were studied (17). In contrast, in patients with acute lymphoblastic leukemia receiving a folate antagonist the presence of the c.677 T/T genotype is associated

with significantly lower rates of grade 3 leukopenia (49, 50). However, larger studies are required to establish the true significance of these observations.

Few studies have addressed the possible importance of *MTHFR* SNPs in predicting clinical outcomes to fluoropyrimidine treatment, and again where published, the data has produced conflicting results (17, 18, 39, 51). We found no associations between *MTHFR* SNPs or diplotypes and clinical response. In terms of survival, *MTHFR* c.677 T/T patients tended to have a shorter survival compared with patients with C/T and C/C genotypes. This is not consistent with *in vitro* findings that suggest patients with the T/T genotype would have reduced *MTHFR* activity compared with those with C/C and C/T genotypes, potentiating the antitumoral effects of 5-FU. As previously suggested, assuming the functional effect of the *MTHFR* c.677 T allele, another possible explanation for our divergent results may be that the cellular availability of 5,10-MTHF may depend not only on *MTHFR* genotype but also other cofactors such as dietary folate (42, 52). Furthermore, population-based studies report an increased risk of malignancy and worse survival outcome in patients with the T/T genotype. It is possible therefore that the T/T genotype is an independent adverse prognostic factor independent of capecitabine therapy. This requires confirmation in a larger study.

We investigated the influence of *MTHFR* haplotype on treatment outcomes to capecitabine therapy in patients with advanced colorectal cancer. This is an important consideration as although *MTHFR* c.1298, like c.677, is associated with lower MTHFR enzymatic activity, *MTHFR* c.1298 alone is not consistently associated with lower plasma folate levels and nor is it reported to be predictive of tumor response to fluoropyrimidines (53). However, compound heterozygosity with the c.677 T allele may affect plasma homocysteine and folate levels, resulting in altered clinical outcome (54). Therefore, a rationale exists to consider the *MTHFR* haplotype in relation to treatment outcomes to fluoropyrimidine-based therapies (55, 56). Consistent with the results for c.677 and c.1298 genotypes, we found the C-C haplotype (c.677C>T-c.1298A>C) seemed to elevate the risk of grade 2/3 toxicity, whereas the T-A/T-A diplotype was protective. Diplotype was not related to response rate or overall survival to capecitabine therapy. This contrasts with the findings of Terrazzino and colleagues (54) who studied the effect of haplotypes on clinical response in patients receiving preoperative 5-FU–based chemotherapy and external beam radiotherapy for rectal cancer. They reported that the T-A haplotype was associated with a lower response rate than other haplotypes. Although this conflicting result may be due to the involvement of radiation, these differences may simply reflect the relatively small number of patients involved and the relatively low rates of antiproliferative toxicities produced by modern infusional schedules of 5-FU and capecitabine.

A number of factors can contribute to inconsistent findings among association studies. These include the type and dose of TS inhibitor administered, cancer type, the stage and grade of the tumor, patient ethnicity, limited sample sizes, misclassification of phenotype, and population structure (57). Cohort sizes in published studies are small, and owing to the frequencies of *MTHFR* c.677 T and c.1298 C alleles, only a limited number of patients homozygous for these alleles have been studied. It is therefore possible that the previous studies lacked adequate power to show an effect of genotype on toxicity, or that the phenotype effect sizes of the *MTHFR* genotypes have been overestimated in our study. Despite these limitations, the findings of this study are novel and may potentially be used as predictors of toxicity before the administration of capecitabine for metastatic colorectal cancer. The results of haplotype-toxicity associations in this circumstance warrant further investigation particularly in the adjuvant setting where variability in efficacy might be more important and in populations of different ethnic groups where toxicities might be more significant.

The second aim of this study was to assess the influence of common *TYMS* and *MTHFR* polymorphisms on folate status, and whether the polymorphisms in association with analyte levels were predictive of toxicity to capecitabine chemotherapy. We found *MTHFR* c.677 T/T patients had lower serum folate and elevated homocysteine concentrations than patients with C/T and C/C genotypes ($P < 0.05$). This finding is consistent with previous studies and is postulated to result from a redistribution of folates (58, 59). We also found that the *MTHFR* c.1298 A/A genotype tended to have elevated homocysteine and vitamin B12 compared with the A/C and C/C genotypes, whilst serum folate did not differ between c.1298 genotypes ($P < 0.01$). The biochemical effect of c.1298A>C is unclear with initial *in vitro* studies, suggesting reduced *MTHFR* activity in the c.1298 variant allele, and subsequent studies showing minimal effect on folate status (15, 60). More recently, the c.1298 C allele has been reported to result in elevated red cell folate as it converts 5,10-MTHF to the more stable 5-methyltetrahydrofolate at an accelerated rate, which may in part explain our results (61). It is also possible that the observed association between c.1298 genotype and homocysteine is a consequence of an indirect association between c.677 genotype, and homocysteine because of the strong linkage disequilibrium between the two SNPs (62). We are the first to examine the influence of *MTHFR* diplotypes on circulating analyte levels in malignancy, and we report that the T-A/T-A diplotype (c.677C>T-c.1298A>C) was associated with higher homocysteine and lower serum folate levels compared with other diplotypes ($P < 0.05$).

We found that *TYMS* genotypes were not associated with folate, homocysteine, or vitamin B12 levels. Similarly, Brown and colleagues (63) did not report any association between TSER genotype and homocysteine levels. This is contrary to two other articles in Chinese populations that suggested a predictive role for TSER in determining red cell folate and homocysteine, and may be attributable to differences in the population studied (8, 64). The *TYMS* 3′UTR polymorphism did not predict red cell folate or homocysteine levels, however on subgroup analysis the indel predicted analyte concentrations in nonsmokers (21). There is a documented association between smoking and a low folate/high homocysteine phenotype that is generally attributed to lower dietary intake, altered absorption, or increased oxidative catabolism of folate but this was beyond the scope of this article (65, 66).

There is increasing evidence for the role of folate status in the development of certain malignancies such as colorectal cancer and acute lymphoblastic leukemia (67, 68). There is also an increasing awareness of the role of pretreatment analyte concentrations in predicting outcomes to folate antagonists (22, 23). Elevated levels of homocysteine have shown to be associated with worse toxicity in patients treated with antifolate

drugs (23). We anticipated that low homocysteine, an indicator of high levels of circulating folate and low intracellular 5,10-MTHF, would be associated with reduced TS inhibition and therefore reduced toxicity; however, we did not observe any relationship between the analytes studied and toxicity. Our contrary results may be explained by the use of multiple imputation method in generating missing data which assumes that data is missing at random and uses the extremes of available data to generate missing data (69). Furthermore, the limited sample size of our study may have limited our ability to explore these relationships in further.

Our preliminary results are interesting in that we have prospectively examined the influence of two genes involved in 5-FU cytotoxicity, as well as the interplay of other potential biomarkers on capecitabine toxicity. Taken together, the data suggests that common genetic variation in *MTHFR* but not *TYMS* may be useful for predicting toxicity from capecitabine. In addition, *MTHFR* SNPs predicted serum folate and plasma homocysteine levels. Confirmatory studies are needed to establish the role of *MTHFR* SNPs and folate status on capecitabine toxicity and efficacy. It is also hoped that the present pilot study will stimulate such studies, particularly as part of large clinical trials, which could eventually result in a strategy for selecting patients with advanced colorectal cancer based on pharmacogenetic analysis.

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