

# Polymorphisms of *MTHFR* C677T and A1298C associated with survival in patients with colorectal cancer treated with 5-fluorouracil-based chemotherapy

Chih-Ching Yeh<sup>1,2</sup> · Ching-Yu Lai<sup>1</sup> · Shih-Ni Chang<sup>3,4</sup> · Ling-Ling Hsieh<sup>5</sup> · Reiping Tang<sup>6,7</sup> · Fung-Chang Sung<sup>3,4</sup> · Yi-Kuei Lin<sup>8</sup>

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## Abstract

**Background** This study examined the association between methylenetetrahydrofolate reductase (*MTHFR*) polymorphisms and survival of patients with colorectal cancer (CRC) treated with 5-fluorouracil (5-FU)-based chemotherapy in Taiwan.

**Methods** We genotyped *MTHFR* polymorphisms C677T (rs1801133) and A1298C (rs1801131) for 498 CRC patients treated with 5-FU-based chemotherapy after receiving surgery. Survival analyses on *MTHFR* polymorphisms were performed using log-rank test and Kaplan–Meier curve. Cox proportional hazards models were used to calculate the hazard ratios (HRs) and 95% confidence intervals (CIs) for the association between *MTHFR* genotypes and survival.

**Results** Overall survival (OS) was significantly longer in CRC patients with *MTHFR* 677 CT+TT genotypes

compared with those with 677 CC genotype (HR 0.77; 95% CI 0.60–0.98). Although the *MTHFR* A1298C polymorphism was not associated with OS in CRC, this polymorphism was associated with significantly shorter OS in rectal cancer. Among rectal cancer patients, OS was shorter for patients with AC+CC genotypes than for those with the AA genotype (HR 1.95; 95% CI 1.35–2.83). In haplotype analysis, better OS was found for colon cancer patients carrying the *MTHFR* 677T-1298A haplotype (HR 0.73; 95% CI 0.55–0.97), but worse survival was linked to rectal cancer patients carrying the *MTHFR* 677C-1298C haplotype (HR 1.53; 95% CI 1.08–2.18).

**Conclusions** Our findings suggest that *MTHFR* genotypes provide prognostic information for CRC patients treated with 5-FU-based chemotherapy.

**Keywords** Colorectal cancer · *MTHFR* · Polymorphisms · Overall survival · 5-Fluorouracil

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✉ Chih-Ching Yeh  
ccyeh@tmu.edu.tw

<sup>1</sup> School of Public Health, College of Public Health, Taipei Medical University, 250 Wu-Hsing Street, Taipei 110, Taiwan

<sup>2</sup> Department of Public Health, College of Public Health, China Medical University, 91 Hsieh-Shih Road, Taichung 404, Taiwan

<sup>3</sup> Management Office for Health Data, China Medical University Hospital, 2 Yude Road, Taichung 404, Taiwan

<sup>4</sup> Graduate Institute of Clinical Medical Science, School of Medicine, College of Medicine, China Medical University, 91 Hsieh-Shih Road, Taichung 404, Taiwan

<sup>5</sup> Department of Public Health, Chang Gung University, 259 Wen-Hwa 1st Road, Guieshan 333, Taoyuan, Taiwan

<sup>6</sup> Colorectal Section, Chang Gung Memorial Hospital, 5 Fusing Street, Guieshan 333, Taoyuan, Taiwan

<sup>7</sup> School of Traditional Chinese Medicine, Chang Gung University, 259 Wen-Hwa 1st Road, Guieshan 333, Taoyuan, Taiwan

<sup>8</sup> Department of Industrial Management, National Taiwan University of Science and Technology, 43, Sec. 4, Keelung Road, Taipei 106, Taiwan

## Introduction

5-Fluorouracil (5-FU), a drug of choice for systemic chemotherapy regimens in colorectal cancer (CRC) patients, exerts its anticancer activity through inhibiting the action of thymidylate synthase (TS) [1]. A stable ternary complex is formed between a 5-FU active metabolite (fluorodeoxyuridine monophosphate), the enzyme and 5,10-methylenetetrahydrofolate (5,10-methyleneTHF) which results in TS inhibition. The optimal inhibition of TS depends on elevated cellular concentrations of 5,10-methyleneTHF.

The concentration of 5,10-methyleneTHF is controlled by the activity of methylenetetrahydrofolate reductase (MTHFR) [2], a critical enzyme that is involved in folate metabolism. This enzyme irreversibly converts 5,10-methyleneTHF to 5-methyltetrahydrofolate (5,10-methylTHF), which is essential for the methylation of homocysteine to methionine [3]. In addition, 5,10-methyleneTHF is also required for conversion of deoxyuridine monophosphate to deoxythymidine monophosphate by TS, which then affects DNA synthesis [4].

Decreased MTHFR enzyme activity results in higher concentrations of 5,10-methyleneTHF that conversely favor the formation and stability of the inhibitory ternary complex. Thus, patients with a genotype associated with decreased MTHFR enzyme activity should be more sensitive to 5-FU than patients with a genotype related to normal MTHFR enzyme expression. MTHFR has several polymorphisms on chromosome 1p [5]. Of these, the 677C>T (Ala to Val) and 1298A>C (Glu to Ala) single nucleotide polymorphisms (SNPs) are the two most commonly connected with altered enzyme activity [6–8]. The 677C>T substitution renders the enzyme thermolabile, and subjects with TT and CT genotypes have approximately 70 and 35% reduced enzyme activity, respectively [6]. The 1298A>C transition also leads to decreased enzyme activity, although not to the same extent as the 677T allele.

Several clinical studies have investigated these two *MTHFR* polymorphisms and clinical outcome in 5-FU-based chemotherapy for CRC, but the data are inconsistent and controversial. Wisotzkey et al. was the first to examine the association between the *MTHFR* C677T polymorphism and clinical response to 5-FU therapy in colon cancer patients, but they were unable to demonstrate an overall survival (OS) difference between patients carrying different *MTHFR* genotypes [9]. In addition, some studies also suggest that *MTHFR* polymorphisms cannot be considered as an independent predictor of clinical outcome in CRC patients receiving 5-FU-based chemotherapy [2, 10–20]. By contrast, many studies have reported an association between *MTHFR* polymorphisms and tumor response to 5-FU-based chemotherapy [21–35].

We conducted a retrospective study to test the hypothesis that *MTHFR* C677T and A1298C polymorphisms are associated with prognosis in CRC patients treated with 5-FU-based chemotherapy. We also explored whether the association differs between colon and rectal cancer.

## Materials and methods

### Patients

The clinicopathological characteristics and chemotherapy regimens of the patients have been clearly described in our previous studies [36, 37]. Briefly, 498 sporadic CRC patients at stages II–IV who received postoperative 5-FU-based chemotherapy as a first-line treatment were enrolled at Chang Gung Memorial Hospital (CGMH) between January 1995 and December 2001 and were followed up every 3–6 months at the outpatient clinic of the Colorectal Section at CGMH. Operative procedures were based on the clinical condition of the patients. We had no predefined criteria of resectability with regard to the tumor characteristics, such as metastatic sites and the number and size of the tumors, etc. All the patients with stages II–III had a curative resection but only 26 (17.1%) patients with stage IV cancer had a curative-intent resection. The study protocol was approved by the Institutional Review Board at China Medical University Hospital (DMR96-IRB-001) and Chang Gung Memorial Hospital (97-0424B). Informed consent was obtained from all individual participants included in the study.

The chemotherapy regimens included oral tegafur–uracil (UFUR) and weekly and monthly intravenous 5-FU plus levamisole or leucovorin (LV) (Supplementary Table S1). The oral tegafur (300–350 mg/m<sup>2</sup>/day) plus levamisole (45 mg/day) regimen was administered daily in 3 doses for 28 days for a 12-month treatment period. The weekly treatment included (1) weekly intravenous bolus injection 5-FU (450 mg/m<sup>2</sup>/day) plus LV (50 mg/day); (2) intravenous injection of 5-FU (450 mg/m<sup>2</sup>/day) weekly plus oral levamisole (50 mg/day) for 3 days every 2 weeks; or (3) a 1-day infusion of high-dose 5-FU (2600 mg/m<sup>2</sup>/day) plus weekly LV (150 mg/day). The monthly treatment included (1) a continuous 5-day infusion of 5-FU (800 mg/m<sup>2</sup>/day) plus LV (50 mg/day) administered once monthly; or (2) a continuous 5-day infusion of 5-FU (800 mg/m<sup>2</sup>/day) administered monthly and combined with oral levamisole (50 mg/day) for 3 days every 2 weeks. The weekly and monthly intravenous medications lasted 12 months for the weekly regimen and 6 months for the monthly regimen. Treatment courses were continuous unless there was evidence of progression, unacceptable toxicity, or the patient

chose to discontinue treatment. Patients were allowed to undergo further chemotherapy at the physician's discretion. The chemotherapeutic toxicity was graded according to the Common Toxicity Criteria of the National Cancer Institute. These toxic events were dichotomously classified for statistical analysis as none-to-moderate (grade 0–2) and severe (grade 3–4).

### Clinical data

Clinical data included medical history, physical examination (rectal and perineal examination), and measurement of carcinoembryonic antigen (CEA) collected during the follow-up period. Liver sonographs, chest X-ray, and colonoscopy (or a barium enema) were performed annually. The criteria for establishing recurrent disease included histological confirmation, or the disease being evident on radiographic or sonographic studies with subsequent clinical progression. We not only collected recurrence and health status of the patient at the follow-up clinical visit but also identified cause of death from the death certificates. The prospective follow-up data were available for all patients until May 2008, with a median follow-up time of 48 months (range 1.5–133 months). Disease-free survival (DFS) was measured from the date of surgery to the time of tumor recurrence, metastasis, or death from any cause. OS was measured from the date of surgery to death from any cause. Patients who were alive at the last follow-up evaluation were censored at that time.

### *MTHFR* polymorphisms

Genomic DNA was extracted from buffy coat cells using standard procedures with sodium dodecyl sulfate (SDS)-proteinase K-RNase digestion and phenol–chloroform extraction. The *MTHFR* C677T (rs1801133) and *MTHFR* A1298C (rs1801131) polymorphisms were detected using polymerase chain reaction (PCR) and restriction fragment length polymorphism methods [6]. All PCR reactions were performed in a 20- $\mu$ l final volume containing 5 pM of each primer, 50 ng genomic DNA, 1.5 mM MgCl<sub>2</sub>, 200  $\mu$ M dNTPs and 1.0 unit of Taq DNA polymerase in the buffer provided by the manufacturer (Fermentas, Glen Burnie, MD, USA). PCR amplification was performed in a Mastercycler gradient thermocycler (Eppendorf, Hamburg, Germany).

The *MTHFR* C677T polymorphism was determined using the following primers: sense, 5'-TGA AGG AGA AGG TGT CTG CGG GA-3'; antisense, 5'-AGG ACG GTG CGG TGA GAG TG-3'. The PCR program was a 2-min denaturing step at 94 °C, followed by 30 cycles of 30 s at 94 °C, 30 s at 60 °C and 30 s at 72 °C. The 198 bp PCR product was digested with *Hinf*I (New England

BioLabs, Beverly, MA): the T allele was cut into 175 and 23 bp fragments; the C allele was not digested. The *MTHFR* A1298C polymorphism was determined using the following primers: sense, 5'-ATG TGG GGG GAG GAG CTG AC-3'; antisense, 5'-GTC TCC CAA CTT ACC CTT CTC CC-3'. The PCR program was a 5-min denaturing step at 92 °C, followed by 35 cycles of 60 s at 92 °C, 30 s at 60.5 °C and 30 s at 72 °C. The 241 bp PCR product was digested with *Mbo*II (Fermentas): the A allele was cut into 204 and 37 bp fragments; the C allele was not digested. For quality control, 10% of randomly selected samples were repeated and showed 100% concordance for both SNPs.

### Statistical analysis

We compared the distributions of each categorical variable by chi-squared test and of continuous variables by Student's *t* test. A univariate analysis of Kaplan–Meier estimates and log-rank test were used to compare survival curves. Cox proportional hazards regression models were used to estimate the hazard ratios (HRs) and their 95% confidence intervals (CIs) on survival according to different genotype. Covariates, such as age, gender, TNM stage and tumor subsite, were included in the multivariate Cox proportional hazards models to estimate adjusted HRs and 95% CIs. HRs for survival risk differences by tumor subsite were estimated using stratification analysis. All analyses were performed using an SAS statistical package (version 9.4 for Windows; SAS Institute, Cary, NC, USA), and two-sided *P* values of <0.05 were considered statistically significant. Haplotypes were constructed and analyzed using THESIAS program (JAVA version) [38].

## Results

### Patient characteristics

The clinicopathological characteristics of the CRC patients are summarized in Table 1. The total of 498 patients included 287 colon cancer patients and 211 rectal cancer patients with a mean age of 57.2 and 60.5 years, respectively (*P* = 0.003). The percentages of stage III and moderately differentiated tumors were significantly higher in rectal cancer patients (61.6 and 84.3%, respectively) than in colon cancer patients (49.5 and 74.0%, respectively). The distributions of sex, family history of cancer and CEA were not different between colon and rectal cancer patients. The genotypic distributions of the two *MTHFR* polymorphisms for CRC patients are also shown in Table 1. The frequencies for the *MTHFR* 677T and *MTHFR* 1298C alleles were 28.5 and 23.5%, respectively. The distributions of the two *MTHFR* polymorphisms were not significantly different

**Table 1** Distribution of clinicopathological characteristics and *MTHFR* polymorphisms of colorectal cancer patients by tumor site

Variables	Total N (%)	Colon N (%)	Rectum N (%)	<i>P</i> <sup>a</sup>
Age (mean ± SD)	58.6 ± 12.4	57.2 ± 12.9	60.5 ± 11.5	0.003
Sex				
Male	263 (52.8)	143 (49.8)	120 (56.9)	0.12
Female	235 (47.2)	144 (50.2)	91 (43.1)	
Multiplicity				
No	474 (95.2)	268 (93.4)	206 (97.6)	0.03
Yes	24 (4.8)	19 (6.6)	5 (2.4)	
Family history of cancer				
No	310 (64.6)	178 (64.7)	132 (64.4)	0.94
Yes	170 (35.4)	97 (35.3)	73 (35.6)	
Missing	18			
TNM stage				
II	74 (14.9)	56 (19.5)	18 (8.5)	0.001
III	272 (54.6)	142 (49.5)	130 (61.6)	
IV	152 (30.5)	89 (31.0)	63 (29.9)	
Histological differentiation				
Well differentiated	61 (12.4)	39 (13.7)	22 (10.5)	0.01
Moderately differentiated	387 (78.3)	210 (74.0)	177 (84.3)	
Poorly differentiated	46 (9.3)	35 (12.3)	11 (5.2)	
Missing	4			
Carcinoembryonic antigen				
<5 ng/ml	224 (46.8)	120 (43.5)	104 (51.2)	0.09
≥5 ng/ml	255 (53.2)	156 (56.5)	99 (48.8)	
Missing	19			
<i>MTHFR</i> C677T				
CC	242 (48.6)	137 (47.7)	105 (49.8)	0.89
CT	228 (45.8)	134 (46.7)	94 (44.5)	
TT	28 (5.6)	16 (5.6)	12 (5.7)	
<i>MTHFR</i> A1298C				
AA	294 (59.0)	171 (59.6)	123 (58.3)	0.96
AC	174 (34.9)	99 (34.5)	75 (35.5)	
CC	30 (6.0)	17 (5.9)	13 (6.2)	

<sup>a</sup> Chi-squared test or *t* test *P* value

between colon and rectal cancer patients even when stratified by tumor stage (Supplementary Table S2).

### Association between *MTHFR* genotype and toxicity

Table 2 shows the incidence of severe toxicity (grade 3–4) by *MTHFR* polymorphisms and tumor site. The percentages of severe leukopenia were significantly higher in patients carrying the *MTHFR* 677 TT genotype (7.1%) than in those carrying the CC or CT genotype (0.8 and 0.9%, respectively) among all CRC patients ( $P = 0.01$ ), as well

as among colon cancer patients ( $P = 0.003$ ). However, the *MTHFR* A1298C polymorphism was not associated with chemotherapeutic toxicity.

### Association between *MTHFR* genotype and DFS

Survival analyses by Kaplan–Meier estimates and log-rank test found no significant association between the two *MTHFR* polymorphisms and DFS (data not shown). In addition, Cox proportional hazards model with an adjustment for age, gender, TNM stage, and tumor subsite showed that the *MTHFR* polymorphisms were not associated with DFS. Stratified analyses by tumor sites for DFS of colorectal cancer associated with *MTHFR* polymorphisms also showed no significant association.

### Association between *MTHFR* genotype and OS

For all CRC patients, the *MTHFR* 677 TT genotype and CT+TT genotype were associated with better OS than the CC genotype (HR 0.55; 95% CI 0.31–0.98; HR 0.77; 95% CI 0.60–0.98, respectively) (Table 3). The *MTHFR* A1298C polymorphism was not associated with OS.

In stratification analysis for tumor subsite, the Kaplan–Meier survival curve showed a significant association between *MTHFR* A1298C polymorphism and OS in rectal cancer (log-rank test  $P = 0.01$ , Fig. 1d). The multivariable Cox proportional hazards model also showed that rectal cancer patients with *MTHFR* A1298C CC and AC genotypes had 2.08-fold (HR 2.08; 95% CI 0.98–4.43) and 1.93-fold (HR 1.93; 95% CI 1.32–2.84) poorer OS than patients with the AA genotype. Meanwhile, rectal cancer patients carrying the *MTHFR* A1298C C allele (AC+CC) had a shorter survival compared to those with the AA genotype (42.2 vs 53.4 months; HR 1.95, 95% CI 1.35–2.83). Interestingly, an inverse relationship between 1298C allele and survival was prominent in stage IV rectal cancer patients (HR 2.65; 95% CI 1.52–4.60) (Supplementary Table S3). However, the *MTHFR* C677T polymorphism was not associated with OS in rectal cancer (Fig. 1c). In addition, OS in colon cancer patients did not differ between the two *MTHFR* polymorphisms as shown in the survival curves (Fig. 1a, b) or in the Cox hazards models (Table 3).

### Haplotype analysis

We further conducted haplotype analysis for *MTHFR* C677T and A1298C polymorphisms and survival in CRC patients. The univariate Kaplan–Meier survival curve showed significant differences in OS among the 4 haplotypes analyzed in all CRC patients (log-rank test  $P = 0.03$ , Fig. 2a), as well as in colon cancer patients (log-rank test  $P = 0.05$ , Fig. 2b). However, OS in rectal cancer patients

**Table 2** Incidence of severe toxicity (grade 3–4) by *MTHFR* polymorphisms and tumor site

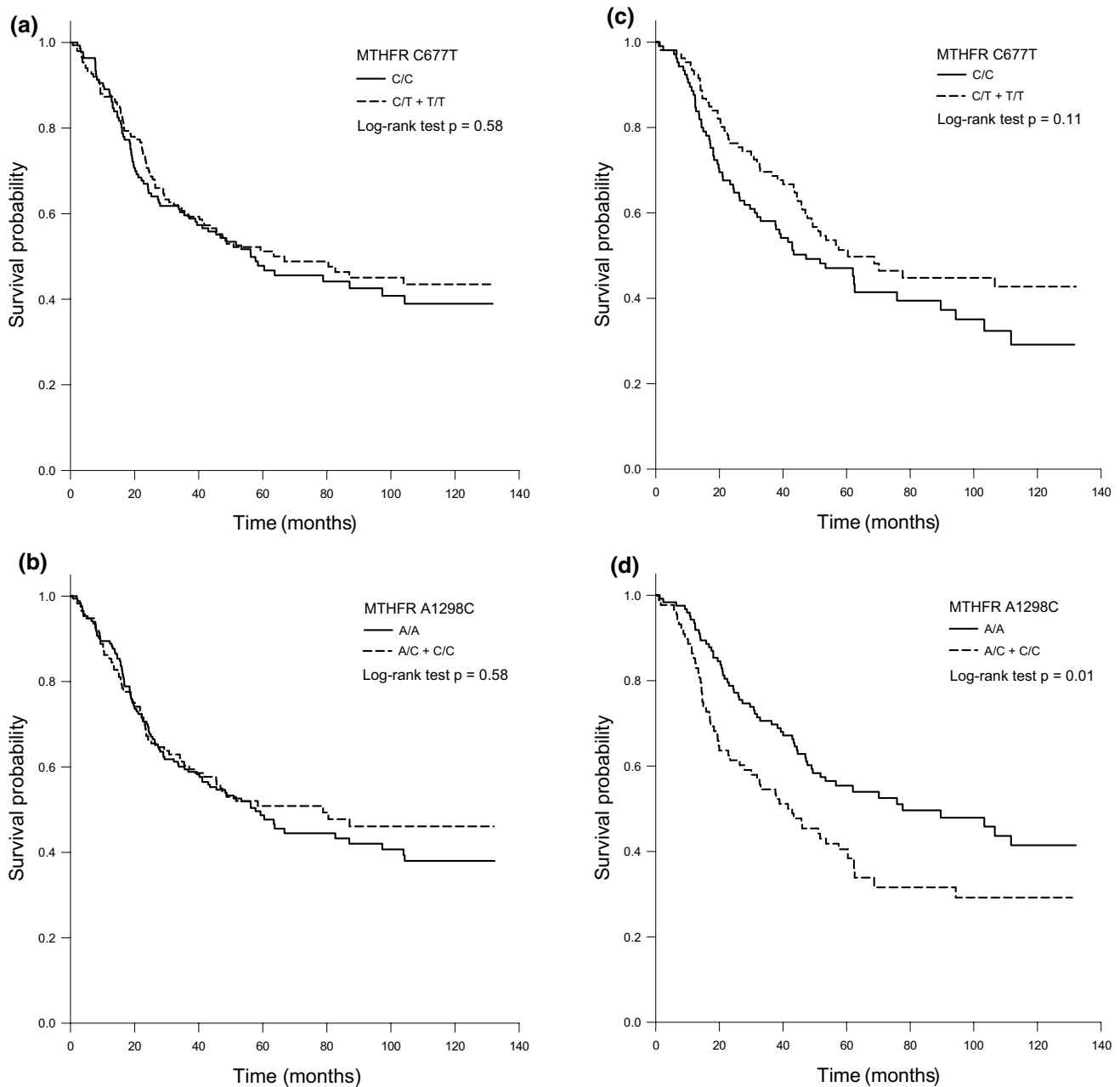
Toxicity, <i>N</i> (%)	C677T			<i>P</i> <sup>a</sup>	A1298C			<i>P</i> <sup>a</sup>
	CC	CT	TT		AA	AC	CC	
Total								
Leukopenia	2 (0.8)	2 (0.9)	2 (7.1)	0.01	4 (1.4)	1 (0.6)	1 (3.3)	0.41
Infection	1 (0.4)	0 (0.0)	0 (0.0)	0.59	0 (0.0)	1 (0.6)	0 (0.0)	0.39
Nausea	1 (0.4)	6 (2.6)	0 (0.0)	0.10	5 (1.7)	2 (1.2)	0 (0.0)	0.71
Vomiting	2 (0.8)	7 (3.1)	0 (0.0)	0.14	8 (2.7)	1 (0.6)	0 (0.0)	0.18
Diarrhea	11 (4.6)	10 (4.4)	2 (7.1)	0.80	15 (5.1)	8 (4.6)	0 (0.0)	0.45
Stomatitis	7 (2.9)	6 (2.6)	1 (3.6)	0.96	11 (3.7)	3 (1.7)	0 (0.0)	0.28
Alopecia	1 (0.4)	1 (0.4)	0 (0.0)	0.94	0 (0.0)	2 (1.2)	0 (0.0)	0.15
Colon								
Leukopenia	1 (0.7)	2 (1.5)	2 (12.5)	0.003	3 (1.8)	1 (1.0)	1 (5.9)	0.37
Infection	1 (0.7)	0 (0.0)	0 (0.0)	0.58	0 (0.0)	1 (1.0)	0 (0.0)	0.39
Nausea	1 (0.7)	3 (2.2)	0 (0.0)	0.51	3 (1.8)	1 (1.0)	0 (0.0)	0.78
Vomiting	0 (0.0)	4 (3.0)	0 (0.0)	0.10	4 (2.3)	0 (0.0)	0 (0.0)	0.25
Diarrhea	4 (2.9)	7 (5.2)	2 (12.5)	0.19	10 (5.9)	3 (3.0)	0 (0.0)	0.37
Stomatitis	5 (3.7)	4 (3.0)	1 (6.3)	0.79	7 (4.1)	3 (3.0)	0 (0.0)	0.65
Alopecia	0 (0.0)	0 (0.0)	0 (0.0)	–	0 (0.0)	0 (0.0)	0 (0.0)	–
Rectum								
Leukopenia	1 (1.0)	0 (0.0)	0 (0.0)	0.60	1 (0.8)	0 (0.0)	0 (0.0)	0.70
Infection	0 (0.0)	0 (0.0)	0 (0.0)	–	0 (0.0)	0 (0.0)	0 (0.0)	–
Nausea	0 (0.0)	3 (3.2)	0 (0.0)	0.15	2 (1.6)	1 (1.3)	0 (0.0)	0.89
Vomiting	2 (1.9)	3 (3.2)	0 (0.0)	0.72	4 (3.3)	1 (1.3)	0 (0.0)	0.58
Diarrhea	7 (6.7)	3 (3.2)	0 (0.0)	0.38	5 (4.1)	5 (6.7)	0 (0.0)	0.50
Stomatitis	2 (1.9)	2 (2.1)	0 (0.0)	0.87	4 (3.3)	0 (0.0)	0 (0.0)	0.23
Alopecia	1 (1.0)	1 (1.1)	0 (0.0)	0.64	0 (0.0)	2 (2.7)	0 (0.0)	0.16

<sup>a</sup> Chi-squared test or Fisher's exact test**Table 3** Cox proportional hazard models of overall survival according to *MTHFR* polymorphisms by tumor site

<i>MTHFR</i>	Total				Colon				Rectum			
	Event	MSM	HR (95% CI) <sup>a</sup>	<i>P</i>	Event	MSM	HR (95% CI) <sup>b</sup>	<i>P</i>	Event	MSM	HR (95% CI) <sup>b</sup>	<i>P</i>
C677T												
CC	138	46.7	1		75	47.1	1		63	42.9	1	
CT	120	48.8	0.80 (0.62–1.02)	0.07	69	47.3	0.80 (0.57–1.11)	0.18	51	51.4	0.80 (0.55–1.15)	0.23
TT	13	48.2	0.55 (0.31–0.98)	0.04	9	48.0	0.64 (0.32–1.29)	0.21	4	57.7	0.42 (0.15–1.15)	0.09
CT+TT	133	48.7	0.77 (0.60–0.98)	0.03	78	47.6	0.77 (0.56–1.07)	0.12	55	52.6	0.75 (0.52–1.07)	0.11
A1298C												
AA	155	48.2	1		94	47.2	1		61	53.4	1	
AC	97	46.4	1.14 (0.88–1.47)	0.32	48	48.7	0.78 (0.55–1.11)	0.17	49	41.6	1.93 (1.32–2.84)	<0.01
CC	19	39.8	1.44 (0.89–2.33)	0.14	11	34.7	1.10 (0.59–2.07)	0.77	8	45.8	2.08 (0.98–4.43)	0.06
AC+CC	116	45.7	1.18 (0.92–1.50)	0.18	59	47.3	0.83 (0.60–1.15)	0.26	57	42.2	1.95 (1.35–2.83)	<0.01

MSM median survival months, HR hazard ratio, 95% CI 95% confidence interval

<sup>a</sup> Adjusted for age, gender, TNM stage, and tumor subsite<sup>b</sup> Adjusted for age, gender, and TNM stage



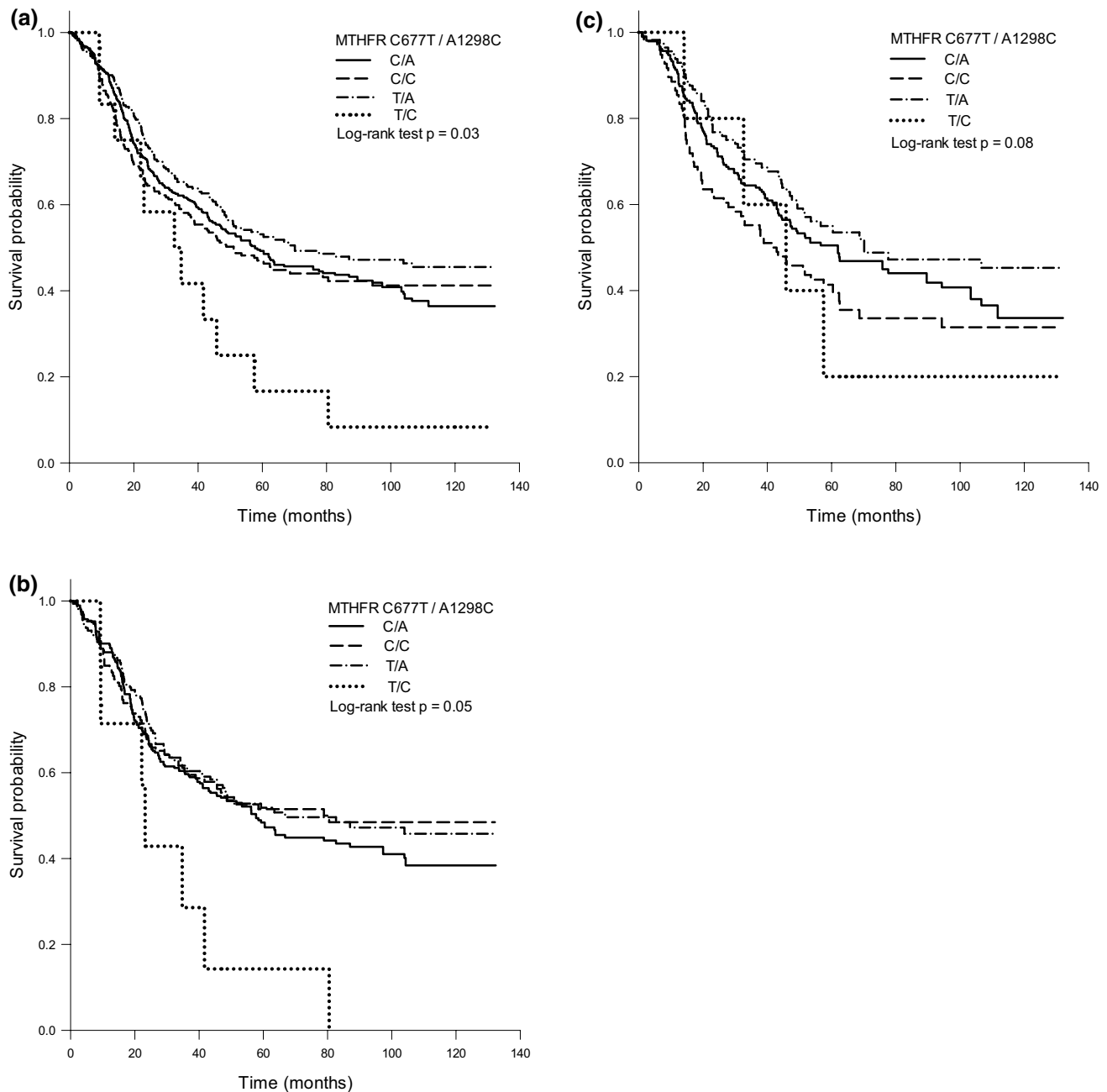
**Fig. 1** Kaplan–Meier analysis and log-rank test of overall survival according to *MTHFR* genotypes in colon cancer patients **a** C677T CC vs CT+TT, **b** A1298C AA vs AC+CC; and in rectal cancer patients **c** C677T CC vs CT+TT, **d** A1298C AA vs AC+CC

did not differ between the 4 haplotypes as shown in Fig. 2c (log-rank test  $P = 0.08$ ).

Table 4 shows the adjusted associations between the *MTHFR* haplotypes and OS in the multivariate Cox proportional hazards models. There was significantly better OS for all CRC patients with the T/A haplotype compared with the most common C/A haplotype (HR 0.77; 95% CI 0.62–0.96), as well as in colon cancer (HR 0.73; 95% CI 0.55–0.97). In contrast, the C/C haplotype was significantly

associated with poor OS in rectal cancer patients (HR 1.53; 95% CI 1.08–2.18). However, the significantly better survival presented in the T/A haplotype disappeared when stratified by tumor stage (Supplementary Table S4). Surprisingly, the worse OS inherited from the C/C haplotype was enhanced in stage IV rectal cancer patients (HR 2.36; 95% CI 1.41–3.95). No association was observed between the *MTHFR* haplotype and DFS in CRC patients even when stratified by tumor subsite.





**Fig. 2** Kaplan–Meier analysis and log-rank test of overall survival according to *MTHFR* haplotypes in **a** all CRC patients, **b** colon cancer patients, and **c** rectal cancer patients

## Discussion

We carried out an analysis of *MTHFR* gene polymorphisms in 498 CRC patients homogeneously treated with 5-FU-based chemotherapy. The results suggested that the *MTHFR* 677T allele could predict better OS in CRC patients treated with 5-FU. Furthermore, the 1298C allele was associated with worse OS in rectal but not in colon cancer cases.

Although several clinical studies have correlated these two *MTHFR* polymorphisms with OS in CRC patients treated with 5-FU-based chemotherapy, the results are inconsistent and contradictory. Some studies have reported that C677T genetic variant is significantly associated with increased tumor response rate to 5-FU-based therapy [21–23, 28]. The 677T allele polymorphic genotype has also been associated with significantly increased time to survival [23, 29, 32, 34, 35], while negative effects on OS

**Table 4** Haplotype analysis for *MTHFR* polymorphisms of survival in colorectal cancer

Haplotype	Total			Colon			Rectum		
	EHF	HR (95% CI) <sup>a</sup>	<i>P</i>	EHF	HR (95% CI) <sup>b</sup>	<i>P</i>	EHF	HR (95% CI) <sup>b</sup>	<i>P</i>
C677T/A1298C									
Disease free survival									
C/A	0.50	1		0.50	1		0.50	1	
T/A	0.27	0.80 (0.61–1.08)	0.15	0.27	0.84 (0.58–1.22)	0.34	0.27	0.78 (0.50–1.22)	0.28
C/C	0.21	0.82 (0.60–1.22)	0.33	0.22	0.66 (0.43–1.00)	0.05	0.21	1.05 (0.65–1.69)	0.83
T/C	0.02	1.59 (0.63–4.01)	0.32	0.01	2.26 (0.50–10.2)	0.29	0.02	1.46 (0.44–4.80)	0.53
Overall survival									
C/A	0.50	1		0.50	1		0.50	1	
T/A	0.26	0.77 (0.62–0.96)	0.02	0.27	0.73 (0.55–0.97)	0.03	0.26	0.81 (0.57–1.15)	0.23
C/C	0.22	1.05 (0.84–1.33)	0.65	0.21	0.77 (0.57–1.05)	0.09	0.22	1.53 (1.08–2.18)	0.02
T/C	0.02	1.15 (0.62–2.14)	0.65	0.02	1.07 (0.48–2.39)	0.87	0.02	1.38 (0.54–3.55)	0.50

EHF estimated haplotype frequency, HR hazard ratio, 95% CI 95% confidence interval

<sup>a</sup> Adjusted for age, gender, TNM stage, and tumor subsite

<sup>b</sup> Adjusted for age, gender, and TNM stage

were reported [27, 31, 33, 39]. On the contrary, the 1298C allele has been correlated with an increased risk of severe adverse events or worse survival after 5-FU-based chemotherapy [22, 25, 27, 28, 34, 35]. However, several other studies do not show any correlation between *MTHFR* polymorphisms and clinical outcome in CRC patients treated with 5-FU-based chemotherapy [2, 9, 10, 15–20, 40–45].

Haplotype analyses have shown that *MTHFR* C677T and A1298C polymorphisms are in high linkage disequilibrium and this approach is also more predictive than the single polymorphism for total plasma homocysteine levels [46]. A few studies have started to investigate the relationship between *MTHFR* haplotype and clinical outcome in CRC patients treated with chemotherapy. Terrazzino et al. conducted a C677T/A1298C haplotype analysis in rectal cancer patients undergoing preoperative 5-FU-based chemoradiation [24], and found that the T/A haplotype predisposed to a worse response and lower tumor regression rate compared to other haplotype combinations. However, using a similar setting, Thomas et al. reported that the C/C haplotype was associated with a protective effect on the incidence of severe diarrhea or mucositis [47]. In our haplotype analysis, the T/A haplotype was significantly related to better OS in CRC patients, while the C/C haplotype was significantly associated with poor OS in rectal cancer patients.

Discrepancies across these studies might occur because of the sample size or ethnic differences in allele frequency for the *MTHFR* polymorphisms. The frequencies for the 677T allele and 1298C amongst the patients in our study (28.5 and 23.5%, respectively) were similar to the results from other studies conducted in Taiwan [14, 34], but appear to be lower than those reported in white American (32.7 and 30.6%) [25], Italian (48.2 and 29.5%) [12], French

(36.1 and 30.4%) [22], or Spanish (39.4 and 30.9%) [10] populations. Moreover, inadequate study design such as a too-small sample size (most are <200) and/or inadequate controlling for certain confounders (such as age and tumor stage) should also be considered as contributors for the differing results.

Possible explanations for divergent findings in clinical studies should also consider the different 5-FU chemotherapy regimens used, i.e., 5-FU-monotherapy (5-FU/LV) or 5-FU-combined chemotherapy (5-FU/irinotecan or 5-FU/oxaliplatin). An ideal model to establish the relationship between *MTHFR* polymorphisms and survival 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, the lack of correlation between *MTHFR* genotypes and survival after 5-FU treatment could result from the effects of an additional drug. Fortunately, we could only include CRC patients treated with a single antineoplastic agent because combined chemotherapy regimens were not supported by the Taiwan Nation Health Insurance before 2000. Our findings are consistent with the correlations found by several investigators in their studies of cancer patients treated with 5-FU-monotherapy [21–23]. Further studies are needed to confirm the role of *MTHFR* genotype and haplotype on survival of CRC patients receiving 5-FU chemotherapy.

Several potential limitations of our retrospective study should be discussed. First, as all patients included in this study presented at stage III, stage IV or high-risk stage II disease, it was not possible to assess genotype associated with clinical outcome in untreated patients. Second, our findings are based on a study population uniformly treated



with 5-FU-based chemotherapy. Although new chemotherapy combinations, such as FOLFOX or FOLFIRI, have been shown to have superior effectiveness and are considered as the standard treatment for CRC patients, these chemotherapies with oxaliplatin or irinotecan could blur the influence of *MTHFR* polymorphisms on the treatment outcomes. Third, our study did not include other genetic polymorphisms in 5-FU-metabolizing enzymes that might be relevant, such as dihydropyrimidine dehydrogenase, TS, MTR, or ABCG2. We expect to extend our investigations to other relevant candidate polymorphisms. Fourth, all the CRC patients are of Chinese ethnicity. Hence, the ability to generalize the results to other racial/ethnic groups awaits further study. Nevertheless, our study which included newly diagnosed and histologically confirmed CRC patients and a relatively large sample size had sufficient power to yield important insights into the overall value of *MTHFR* genotypes as a prognostic marker.

To the best of our knowledge, this is one of the largest studies to show a relationship between two *MTHFR* polymorphisms and OS in CRC patients treated with 5-FU-based chemotherapy in a Chinese population. Our findings suggest that the *MTHFR* 677T allele exerts a protective role in OS in CRC patients, while the *MTHFR* 1298C allele may have a harmful effect on OS in rectal cancer. Further prospective studies measuring *MTHFR* gene expression related to its genotype are warranted to understand the role of *MTHFR* polymorphisms as a predictor of chemotherapeutic benefit in CRC patients.

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#### Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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