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The effects of genomic polymorphisms in one-carbon metabolism pathways on survival of gastric cancer patients received fluorouracil-based adjuvant therapy

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5-fluorouracil (5-FU) is widely used to treat patients with gastric cancer (GC). However, the response rate is quite heterogeneous. The single nucleotide polymorphisms (SNPs) and their interactions of genes in the one-carbon metabolism (OCM) pathway, including Methylenetetrahydrofolate reductase (*MTHFR*), Methionine synthase reductase (*MTRR*), Methionine synthase (*MTR*), and Thymidylate synthase (*TS*), significantly affect 5-FU metabolism. In this study, 650 stage II-III patients were recruited from 1998 to 2006. Among them, 251 received 5-FU treatment and other 399 patients were untreated. The Cox regression analysis, log-rank tests and Kaplan–Meier plots were adopted. In the chemotherapy cohort, *MTRR* 66 GA + GG genotypes decreased death risk, however, the protect effect of *MTRR* 66 GA + GG disappeared when GC patients simultaneously had *MTHFR* 677TT + TC or *MTR* 2756GG + GA genotypes. *TS* 5'-UTR 2R3R + 3R3R genotypes also prolonged overall survival of patients treated with 5-FU. And this favorable prognosis obviously enhanced when GC patients simultaneously had *TS* 3'-UTR DD + DI and *TS* 5'-UTR 2R3R + 3R3R genotypes. Our findings showed that the polymorphisms of *MTRR* 66 A > G and *TS* 5'-UTR 3R > 2R may be potential prognostic factors for GC patients receiving 5-FU.

Despite annual decline in death rates of the gastric cancer (GC) over the past decade, it is still the second most common cause of cancer death worldwide¹. China possesses 47% of new global GC cases, with the highest mortality rate among all malignant cancers². Two thirds of GC patients at early or advanced stage recur after curative resection. So, the first line of treatment is chemotherapy followed by surgery for most GC patients³. The combination of surgery with chemotherapy obviously improved 5-year survival rates from 49.6% to 55.3% compared with surgery alone⁴. Although new anticancer drugs have been actively developed, 5-fluorouracil (5-FU) still remains one of the most widely used regimes for the treatment of stage II-III GC patients⁵. However, the response rate is only approximately 25%, even if supplemented by leucovorin (CF), which improves the effects of 5-FU⁶. Single-nucleotide polymorphisms (SNPs) of various genes involved in the one-carbon metabolism pathway may play a pivotal role in the efficacy of 5-FU for stage II-III GC patients. Therefore, it is of great importance to identify new biomarkers that can be used as predictors for 5-FU-based chemotherapy.

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One-carbon metabolism (OCM) pathway plays an important role in the processes of DNA methylation and DNA synthesis. The enzymes, including *Methionine synthase reductase (MTRR)*, *Methionine synthase (MTR)*, *Methylenetetrahydrofolate reductase (MTHFR)*, and *Thymidylate synthase (TS)*, are key components to catalyze S-adenosylmethionine (SAM) synthesis from folate update⁷. The antitumor effect of 5-FU depends on an interaction with key enzymes in OCM pathway⁸. As a pyrimidine analogue, 5-FU is metabolized to 5-fluoro-2'-deoxyuridine 5'-monophosphate (FdUMP), which inhibits thymidylate synthase (TS) by forming a ternary complex involving FdUMP, 5,10-methylene tetrahydrofolate (5,10-MTHF) and TS⁹. TS catalyzes methylation of dUMP to dTMP and this is an important process in DNA synthesis¹⁰. This ternary complex inhibits TS activity and suppresses DNA synthesis, which is thought to be the main mechanism of the antitumor effect of 5-FU⁸. The stabilization and formation of this complex relays on the concentration of 5,10-MTHF. Methylenetetrahydrofolate reductase (MTHFR) catalyzes an irreversible conversion of 5,10-MTHF to 5-methyltetrahydrofolate(5-MTHF)¹¹. Reduced enzyme activity of MTHFR lead to an increased concentration of 5,10-MTHF and augment the sensitivity of 5-FU¹². Until now, 5-FU based adjuvant chemotherapy is widely considered to be the traditional treatment regimen for GC, leading to improvement of quality of life, overall survival (OS) and progression free survival (PFS)¹³. The 5-FU-based regimen normally combines 5-FU with cisplatin (DDP) or oxaliplatin (L-OHP).

The polymorphisms of OCM associated genes may be crucial predictive factors for the GC patients treatment with 5-FU. Two polymorphisms have been identified in TS, including two or three 28-bp repeated (2R/3R) sequences in the 5'-untranslated region (UTR) and a 6-bp insertion/deletion (I/D) in the 3'-UTR^{14,15}. The 3R allele could enhance TS expression when compared with the 2R allele, and the TS 3'-UTR del6 allele is associated with decreased TS mRNA stability and lower TS expression in comparison with the ins6 allele^{15,16}. The SNPs of TS influence the response rate to 5-FU by regulating TS expression levels. The MTHFR 677 C > T (rs1801133, Ala222Val) and 1298 A > C (rs1801131, Glu429Ala) polymorphisms are common gene variants that decrease MTHFR activity^{17,18}. Reduced MTHFR activity increases the accumulation of 5,10-MTHF, which may increase the cytotoxic effect of 5-FU by stabilization of the ternary complex with TS and FdUMP⁹.

Methionine synthase (MTR) catalyzes the transfer of the methyl base from 5-methyl-tetrahydrofolate (5-MTHF) to homocysteine, producing methionine and THF. The function of methionine synthase reductase (MTRR) is to maintain MTR activity¹⁹. The SNP of MTR 2756 A > G, (rs1805087, Asp868Gly) and MTRR 66A > G, (rs1801394, Ile22Met) may decrease methionine and elevate 5-MTHF levels, thereby increasing the concentrations of 5,10-MTHF²⁰. Therefore a decreased activity of the MTR and MTRR would improve the treatment effect of 5-FU-based chemotherapy.

Patients with stage II-III GC are the major group to be dedicatedly cared for anticancer therapy in clinic. Although the effects of SNPs of individual gene or combinations of two genes on the survival benefited from 5-FU have been reported, the synergistic effects of the OCM pathway associated genes polymorphisms on stage II-III GC patients have not been investigated. Based on large-number clinical data analysis, our study systematically investigated the individual and synergistic effects of polymorphisms of TS, MTHFR, MTR and MTRR on the prognosis of stage II-III GC patients accepted 5-FU-based adjuvant chemotherapy.

Results

Clinical features of the study patients. The clinical features of 650 enrolled patients were summarized in Table 1. The median age was 61.0 years (range, 28–83 years) and 328 of them died during the follow-up of 119.0 months. 251 patients underwent 5-FU-based adjuvant chemotherapy and 399 of them did not receive chemotherapy after surgical resections. In the no adjuvant chemotherapy cohort, the survival time was significantly related to varieties in tumor differentiation, lymph node metastasis and TNM stage (all log-rank $p < 0.05$). In particular, no chemotherapy patients with III level of TNM stage (MST, 36 months) had a 80.9% notably higher risk of death (HR = 1.809, 95% CI = 1.322–2.477, $p < 0.001$) when compared with those with II level (MST, 71 months). Patients with lymph node metastasis (MST, 38 months) had a 50.0% higher risk of death (HR = 1.500, 95% CI = 1.056–2.129, $p = 0.021$) when compared to those without lymph node metastasis (MST, 67 months). Meanwhile, patients with well differentiation (MST, 74months) had 43.6% and 67.5% lower risk of death when compared to those with poorly differentiation (MST, 38 months; HR = 1.436, 95% CI = 1.037–1.989) and mucinous or signet-ring cell (MST, 35 months; HR = 1.675, 95% CI = 1.064–2.636; $p = 0.038$), respectively. In contrast, no remarkable higher death risks were founded among the chemotherapy cohort.

MTRR 66A > G and TS 5'-UTR 2R > 3R polymorphism prolonged overall survival of GC patients receiving 5-FU-based adjuvant chemotherapy.

Cox regression analyses were used to assess relationships of polymorphisms of those genes in the OCM pathway with overall survival in different genetic models among the two cohorts (Table 2). For the patients with adjuvant regimens, significant associations were found between MTRR 66A > G polymorphism and overall survival time in the dominant model. Chemotherapy patients with MTRR 66GA + GG genotypes (MST, 80 months) exhibited a 34.3% obviously lower risk of death (HR = 0.657, 95% CI = 0.446–0.967, $p = 0.031$, Table 2, Fig. 1) when compared with the patients with AA genotype (MST, 46 months), whereas no associations were founded among the cohort without chemotherapy.

For TS 5'-UTR 2R > 3R polymorphism, chemotherapy patients with 2R3R + 3R3R genotype (MST, 73 months) had a 50.2% notably lower risk of death (HR = 0.498, 95% CI = 0.259–0.960, $p = 0.032$, Table 2) when compared with those with 2R2R genotype (MST, 23 months). 5'-UTR 3R3R genotype alone also remarkably prolonged the survival time of chemotherapy patients (MST, 70 months; HR = 0.435, 95% CI = 0.221–0.857, $p = 0.024$).

For other polymorphisms including MTHFR 677C > T, 1298A > C MTR 2756A > G and TS3'-UTR 6 bp ins > del, no significant relationships were founded between polymorphisms in any genetic models and survival

Variable	Chemotherapy (251)				No chemotherapy (399)			
	Patients/deaths	MST (months)	P	HR (95% CI) ^a	Patients/deaths	MST (months)	p	HR (95% CI) ^a
age(years)								
<=60	144/60	67 ^b	0.264	1	161/90	43	0.888	1
>60	107/50	50		1.236 (0.849–1.801)	138/128	50		1.020 (0.777–1.337)
Sex								
Male	205/91	70 ^b	0.694	1	301/159	50	0.304	1
Female	46/19	55		0.906 (0.553–1.486)	98/59	36		1.168 (0.866–1.576)
Tumor size								
<=5	141/59	73 ^b	0.304	1	211/111	53	0.223	1
>5	110/51	55		1.215 (0.835–1.768)	188/107	47		1.178 (0.903–1.537)
Tumor site								
No-cardia	157/69	64 ^b	0.782	1	253/142	38	0.386	1
Cardia	94/41	55		0.947 (0.644–1.394)	146/76	59		0.885 (0.669–1.170)
Histological type								
Intestinal	73/35	62	0.417	1	143/75	42	0.306	1
Diffuse	178/75	72 ^b		1.179(0.789–1.762)	256/143	54		1.158(0.872–1.537)
Tumor differentiation								
Well	68/33	51	0.148	1	120/50	74	0.038	1
Poorly	151/59	75 ^b		0.742 (0.484–1.137)	231/137	38		1.436 (1.037–1.989)
Mucinous or signet-ring cell	32/18	45 ^b		1.171(0.659–2.079)	47/30	35		1.675(1.064–2.636)
Depth of invasion								
T1/T2	24/13	35	0.390	1	43/19	68 ^b	0.168	1
T3/T4	227/97	72 ^b		0.778 (0.436–1.388)	351/195	48		1.388 (0.866–2.226)
Lymph node metastasis								
NO	52/18	69 ^b	0.101	1	88/38	67	0.021	1
N1/N2/N3	199/92	62		1.518 (0.915–2.517)	308/178	38		1.500 (1.056–2.129)
TNM stage								
II	72/29	64 ^b	0.325	1	126/51	71 ^b	<0.001	1
III	179/81	69 ^b		1.235 (0.808–1.888)	273/167	36		1.809 (1.322–2.477)

Table 1. Characteristics of the two cohorts of the gastric cancer patients. ^aAdjusted for age and sex. ^bMean survival time was provided when MST could not be calculated. ^cInformation was not available for two patients. HR, hazard ratio; CI, confidence interval; MST, median survival time; P, Log-rank p.

among patients accepted 5-FU treatment (Table 2). Furthermore, none of the six polymorphisms were correlated with overall survival for patients without 5-FU treatment.

Stratified analyses of MTRR 66A > G polymorphism on reducing the death risk of GC patients accepted 5-FU-based chemotherapy.

To further explore the relationships between survival time and MTRR 66A > G polymorphism among GC patients with 5-FU-based chemotherapy, stratified analysis were conducted according to the histological types, sizes, sites, TNM stages, depth of invasion, lymph node metastasis, distant metastasis and chemotherapy (Table 3). Compared with the AA genotype, the MTRR 66 GA + GG genotypes were obviously associated with favorable survival of GC patients in diffuse type (MST, 83 months vs. 43 months; HR = 0.573; 95% CI: 0.356–0.923, p = 0.019), poorly differentiation (MST, 89 months vs. 50 months; HR = 0.460; 95% CI = 0.266–0.795, p = 0.004), T3/T4 level depth of invasion (MST, 82 months vs. 50 months; HR = 0.631, 95% CI = 0.414–0.961, p = 0.029), N1/N2/N3 lymph node metastasis (MST, 80 months vs. 39 months; HR = 0.562, 95% CI = 0.367–0.863, p = 0.007) and III level TNM stages (MST, 81 months vs. 39 months; HR = 0.557, 95% CI = 0.350–0.885, p = 0.01, Table 3, Fig. 2).

Furthermore, we stratified patients by chemotherapy regimens as combinations of DDP and 5-FU, or L-OHP and 5-FU subgroups. In dominant model, MTRR 66 GA + GG patients treated with L-OHP and 5-FU had favorable prognosis (MST, 92 months vs. 62 months; HR = 0.538, 95% CI = 0.299–0.970, p = 0.035, Table 4, Fig. 3) than those with AA genotype. Markedly, the death rate was reduced from 41.5% (27/65, patients with AA genotype) to 25.3% (19/75, patients with GA + GG genotype). Surprisingly, no statistically significant protection was observed in subgroup accepted chemotherapy based on DDP and 5-FU (HR = 0.839, 95% CI = 0.506–1.393, p = 0.494, Table 4, Fig. 3).

The effects of combined polymorphisms on 5-FU-based chemotherapy. Considering the complicated interactions of the genes outlined above, we investigated the effect of polymorphism interactions on the prognosis of GC patients who accepted 5-FU treatment (Supplementary Tables S1–S3). In univariate analysis, MTRR 66 GA + GG genotypes remarkably reduced death risk, but the elongated survival time disappeared

Genetic model	Genotypes	Chemotherapy			No chemotherapy			
		MST (months)	P	HR (95% CI) ^a	MST (months)	p	HR (95% CI) ^a	
<i>MTRR rs1801394 66 A > G</i>								
Codominant model	AA	46	0.089	1	38	0.419	1	
	GA	72 ^b		0.675 (0.452–1.007)			62	0.880 (0.659–1.176)
	GG	85 ^b		0.526 (0.192–1.443)			62	0.683 (0.347–1.344)
Dominant model	GA/GG	80 ^b	0.031	0.657 (0.446–0.967)	62		0.853 (0.645–1.129)	
<i>TYMS 5'-UTR 2R > 3R</i>								
Codominant model	2R2R	23	0.024	1	60 ^b	0.600	1	
	2R3R	51		0.629 (0.312–1.268)			50	1.433 (0.658–3.124)
	3R3R	70 ^b		0.435 (0.221–0.857)			50	1.474 (0.687–3.164)
Dominant model	2R3R/3R3R	73 ^b	0.032	0.498 (0.259–0.960)	50	0.320	1.459 (0.685–3.106)	
Recessive model	2R2R/2R3R	39	0.022	1	56	0.643	1	
	3R3R	70 ^b		0.637 (0.430–0.944)			50	1.071 (0.800–1.433)
<i>MTHFR rs1801131 1298 A > C</i>								
Codominant model	AA	62	0.55	1	48	0.739	1	
	CA	72 ^b		1.011 (0.656–1.559)			48	1.012 (0.747–1.371)
	CC	69 ^b		0.471 (0.116–1.918)			68 ^b	0.585 (0.145–2.362)
Dominant model	CA/CC	75 ^b	0.771	0.940 (0.616–1.433)	48	0.934	0.987 (0.732–1.333)	
<i>MTHFR rs1801133 677C > T</i>								
Codominant model	CC	72 ^b	0.697	1	38	0.610	1	
	TC	59 ^b		1.169 (0.771–1.774)			43	0.973 (0.694–1.266)
	TT	55		0.983 (0.555–1.741)			63	0.814 (0.541–1.226)
Dominant model	TC/TT	60 ^b	0.587	1.118 (0.753–1.659)	48		0.904 (0.679–1.203)	
<i>MTR rs1805087 2756 A > G</i>								
Codominant model	AA	62	0.613	1	53	0.059	1	
	GA	73		0.943 (0.559–1.591)			42	1.3699 (0.957–1.958)
	GG	0		0				0
Dominant model	GA/GG	64 ^b	0.681	0.897 (0.532–1.513)	26	0.205	1.257 (0.879–1.797)	
<i>TYMS 3'-UTR 6bp ins > del</i>								
Codominant model	I/I	46	0.171	1	55 ^b	0.811	1	
	D/I	50		1.028 (0.539–1.961)			47	1.207 (0.677–2.151)
	D/D	71 ^b		0.703 (0.359–1.376)			50	1.192 (0.668–2.128)
Dominant model	DI/DD	72 ^b	0.654	0.868 (0.456–1.620)	48	0.521	1.200 (0.684–2.104)	

Table 2. Genotypes of *MTRR*, *MTHFR*, *MTR* and *TS* polymorphism with gastric cancer patients' survival in both cohorts. ^aAdjusted for age and sex. ^bMean survival time was provided when MST could not be calculated. 2R, 2-repeat; 3R, 3-repeat; I, 6-bp insertion; D, 6-bp deletion; P, Log-rank p.

when GC patients simultaneously carried *MTR* 2756GA + GG genotypes (HR = 0.871, 95% CI = 0.443–1.713) or *MTHFR* 677TT + TC genotypes (HR = 0.761, 95% CI = 0.451–1.287, Table 5). In these cases, the MSTs were reduced from 80 months to 60 and 64 months, respectively. In addition, it seemed that patients simultaneous with *TS* 3'-UTR DD + DI and *TS* 5'-UTR 2R3R + 3R3R genotypes showed higher reduction of death risk (HR = 0.332, 95% CI = 0.134–0.822, log-rank p = 0.046, Table 5) than those with *TS* 5'-UTR 2R3R + 3R3R (HR = 0.498, 95% CI = 0.259–0.960) genotypes alone.

Discussion

We systematically investigated the effects of SNPs in the OCM pathway and their interactions on the survival of stage II-III GC patients, who received 5-FU base adjuvant chemotherapy. Our results suggested that the polymorphisms of *MTRR* 66A > G and *TS* 5'-UTR 2R > 3R remarkably prolonged the survival of Chinese stage II-III GC patients treated with 5-FU base regimen. The MST of GC patients was also significantly influenced by interactions between *MTRR* 66A > G, *MTR* 2756A > G, *TS* 5'-UTR 2R > 3R and *MTHFR* 677C > T.

As an inhibitor of DNA synthesis, 5-FU displays potent anticancer activity by inhibiting *TS* activity through forming a ternary complex with 5, 10-MTHF²¹. The SNPs of *MTRR* 66 A > G and *MTR* 2756 A > G has been shown to decrease *MTR* enzyme activity. *MTRR* is used to maintain *MTR* in its active form, but the activity of *MTR* could not be preserved at a high level because the substitution of Ile22 to Met22 in *MTRR* caused by the 66A > G mutation decreased *MTR* activity²². The reduced *MTR* activity resulted in an accumulation of 5-MTHF. Subsequently, it increases the level of 5,10-MTHF that ultimately enhances the effect of 5-FU treatment²⁰. Our result indicated that *MTRR* 66GA + GG genotypes significantly improved the survival of GC patients in the chemotherapy cohort (HR = 0.657, 95% CI = 0.446–0.967, Table 2, Fig. 1). This is consistent with other studies^{23,24}. Further stratified analysis on pathological features showed that many factors, including histological type, tumor differentiation, depth of invasion, lymph node metastasis and TNM stages of patients, strongly influenced

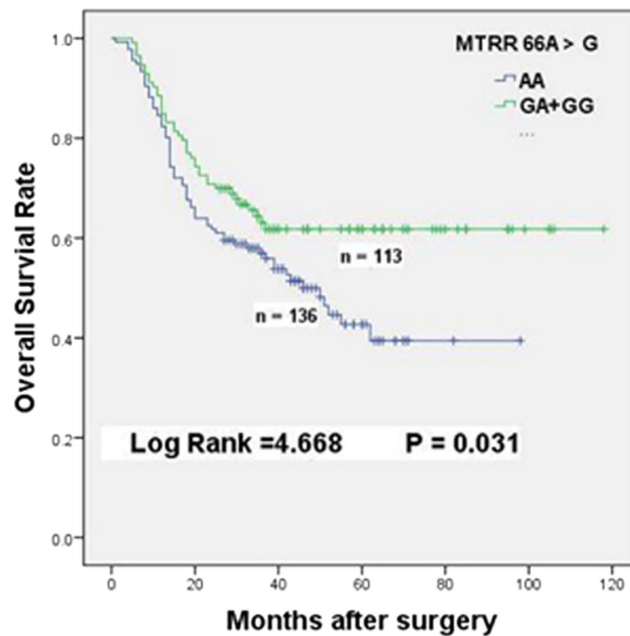


Figure 1. Overall survival of *MTRR66A* > G dominant genotypes in GC patients receiving 5-FU-based chemotherapy.

Variables	Genotypes (Dominant model)		MST (AA/GA + GG)	HR (95% CI) ^a	<i>P</i> _{Heterogeneity}
	AA	GA/GG			
Total	136/69	113/41	46/80 ^b	0.657 (0.446–0.967)	0.031
Age					
≤60	73/35	69/25	52/73 ^b	0.697 (0.416–1.165)	0.163
>60	63/34	44/16	42/79 ^b	0.622 (0.343–1.127)	0.109
Tumor size					
≤5 cm	77/38	64/21	46/84 ^b	0.607 (0.356–1.036)	0.062
>5 cm	59/31	49/20	51/67 ^b	0.725 (0.413–1.273)	0.255
Tumor site					
Non-cardia	88/44	68/25	43/72 ^b	0.645 (0.394–1.054)	0.076
Cardia	48/25	45/16	51/80 ^b	0.700 (0.373–1.311)	0.259
Histological type					
Intestinal type	40/20	32/15	51/58 ^b	0.891 (0.456–1.743)	0.734
Diffuse type	96/49	81/26	43/83 ^b	0.573 (0.356–0.923)	0.019
Tumor differentiation					
Well to moderately	37/19	30/14	46/57 ^b	0.864 (0.433–1.724)	0.676
poor	80/40	71/19	50/89 ^b	0.460 (0.266–0.795)	0.004
mucinous/signet-ring cell	19/10	12/8	43/20	1.514 (0.592–3.871)	0.382
Depth of invasion					
T1/T2	8/5	16/8	24/56 ^b	0.681 (0.222–2.088)	0.495
T3/T4	128/64	97/33	50/82 ^b	0.631 (0.414–0.961)	0.029
Lymph node metastasis					
N0	29/10	22/8	70b/65 ^b	1.276 (0.502–3.244)	0.606
N1/N2/N3	107/59	91/33	39/80 ^b	0.562 (0.367–0.863)	0.007
Distant metastasis					
M0	136/69	113/41	46/80 ^b	0.657 (0.446–0.967)	0.031
TNM stage					
II	36/15	35/14	62/63 ^b	1.073 (0.517–2.226)	0.849
III	100/54	78/27	39/81 ^b	0.557 (0.350–0.885)	0.011

Table 3. Stratified analysis of the *MTRR66A* > G polymorphism with gastric cancer overall survival in patients received adjuvant chemotherapy. ^aAdjusted for age and sex. ^bHeterogeneity test for differences between groups. ^cInformation was not available for two patients.

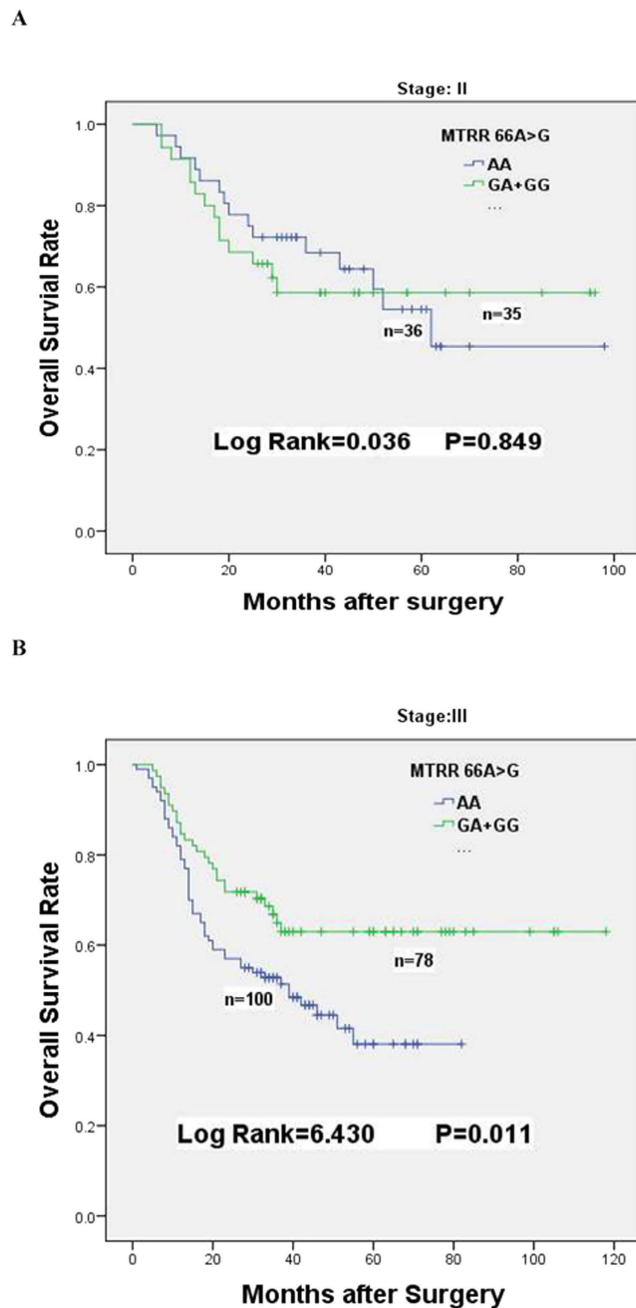


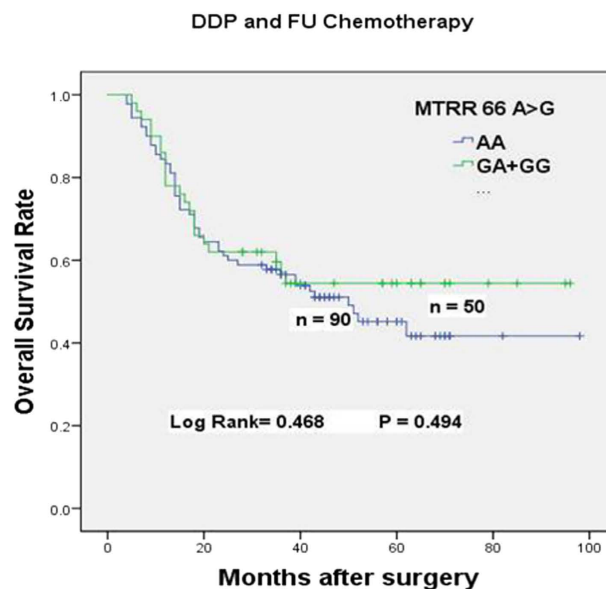
Figure 2. Kaplan-Meier survival curves of *MTRR66A* > *G* dominant genotypes for overall survival of GC patients received adjuvant chemotherapy. (A) Overall survival of *MTRR66A* > *G* dominant genotypes in stage II chemotherapy patients. (B) Overall survival of *MTRR66A* > *G* dominant genotypes in stage III chemotherapy patients.

the survival of GC patients carrying *MTRR* 66GA + GG genotypes (Table 3). In the case of *MTR* 2756 A > G polymorphism, Sarbia *et al.* had indicated that patients with *MTR* 2756GA + GG genotypes had favorable outcomes when they underwent multimodal treatment including 5-FU-based radiochemotherapy and surgery²⁵. However, we found that this polymorphism had no obvious association with prognosis of patients receiving 5-FU-based chemotherapy. In the combination analysis, although the favorable response was maintained when patients simultaneously carry *MTRR* 66GA + GG and *MTR* 2756AA genotypes; interestingly, *MTR* 2756GG + GA genotypes neutralized the protective effect of *MTRR* 66GA + GG (HR = 0.871, 95% CI = 0.443–1.713, Table 5). The similar neutralization was also existed when patients simultaneous with *MTRR* 66GG + GA and *MTHFR* 677TT + TC genotypes (Table 5). The neutralization maybe caused by the interactions of those key enzymes involved in the OCM pathway. Asp868 of *MTR* and Ile22 of *MTRR* may play vital role in the interactions between *MTRR* and *MTR*. Met22 conversion of *MTRR* may breakdown *MTRR*-*MTR* interaction, but Gly868 conversion of *MTR* may compensate and recover those interactions to a certain extent by changing the three dimensional spatial

<i>MTRR66A > G</i>					
Chemotherapy Based on DDP and 5-FU					
Genotype	Patients, n = 140	Deaths, n = 69	MST (months)	log-rank p	HR (95% CI) ^a
AA	90	47	50	0.494	1
GA/GG	50	22	60 ^b		0.839 (0.506–1.393)
Chemotherapy Based on L-OHP and 5-FU					
Genotype	patients, n = 140	Deaths, n = 46	MST (months)	log-rank p	HR (95% CI) ^a
AA	65	27	62	0.035	1
GA/GG	75	19	92 ^b		0.538 (0.299–0.970)

Table 4. Association between the *MTRR 66A > G* dominant model and survival of gastric cancer patients among various chemotherapy regimen subgroups. ^aAdjusted for age and sex. ^bMean survival time was provided when MST could not be calculated.

A



B

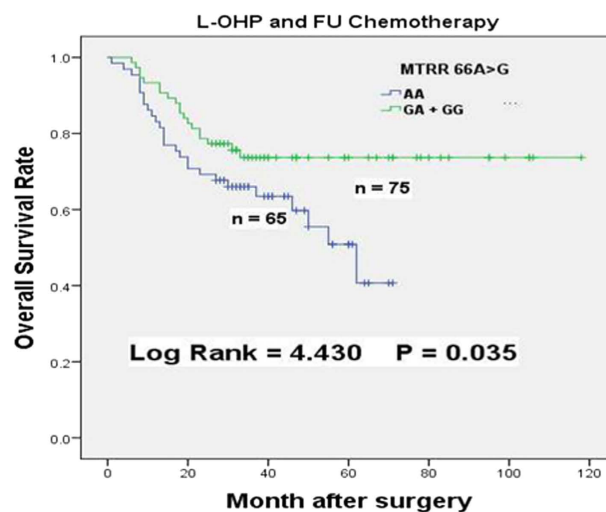


Figure 3. Kaplan-Meier survival curves of *MTRR66A > G* dominant genotypes for overall survival of GC patients received various chemotherapy regimen subgroups. **(A)** Overall survival of *MTRR66A > G* dominant genotypes patients treated with chemotherapy based on DDP and 5-FU. **(B)** Overall survival of *MTRR66A > G* dominant genotypes patients treated with chemotherapy based on L-OHP and 5-FU.

Combined genotypes	Patients	Deaths	MST (months)	P	HR (95%CI) ^a
<i>MTRR 66A > G and MTR 2756A > G</i>					
<i>MTRR 66AA + MTR 2756AA</i>	105	54	43	0.149	1
<i>MTRR 66AA + MTR 2756GG + GA</i>	19	7	65 ^b		0.631 (0.287–1.388)
<i>MTRR 66GG + GA + MTR 2756AA</i>	82	28	81 ^b		0.607 (0.384–0.959)
<i>MTRR 66GG + GA + MTR 2756GG + GA</i>	21	10	60 ^b		0.871 (0.443–1.713)
<i>MTRR 66A > G and MTHFR 677C > T</i>					
<i>MTRR 66AA + MTHFR 677CC</i>	57	27	50	0.133	1
<i>MTRR 66AA + MTHFR 677TT + TC</i>	77	41	43		1.146 (0.705–1.864)
<i>MTRR 66GG + GA + MTHFR 677CC</i>	34	11	85 ^b		0.588 (0.291–1.187)
<i>MTRR 66GG + GA + MTHFR 677TT + TC</i>	77	29	64 ^b		0.761 (0.451–1.287)
<i>TS3-UTR I > D and TS5-UTR 2R > 3R</i>					
<i>TS3-UTR II + TS5-UTR 2R2R</i>	5	5	18	0.046	1
<i>TS3-UTR II + TS5-UTR 2R3R + 3R3R</i>	16	5	72 ^b		0.223 (0.064–0.776)
<i>TS3-UTR DD + DI + TS5-UTR 2R2R</i>	9	5	39		0.481 (0.139–1.666)
<i>TS3-UTRDD + DI + TS5-UTR 2R3R + 3R3R</i>	202	85	73 ^b		0.332 (0.134–0.822)

Table 5. The effects of gene-gene interactions on the survival of gastric cancer patients received adjuvant chemotherapy. ^aAdjusted for age and sex. ^bMean survival time was provided when MST could not be calculated.

structure²⁶. Similar interactions probably also exist between MTRR and MTHFR. Because our results were from a single cohort study with limited number of patients, the results would be validated in other cohorts with enlarged sample sizes.

The stratified analysis of different combined chemotherapy regimens showed that patients with MTRR 66GA + GG genotypes treated with L-OHP and 5-FU had remarkably favorable prognosis than those with AA genotypes (HR = 0.538, 95% CI = 0.299–0.970, $p = 0.035$, Table 4, Fig. 3). Whereas, no obviously improved survival was founded among patients receiving DDP and 5-FU chemotherapy (HR = 0.839, 95% CI = 0.506–1.393, $p = 0.494$, Table 4, Fig. 3). This is not surprising because L-OHP displays much more potent apoptotic activity than DDP²⁷. Although L-OHP forms fewer intrastrand cross-links with adjacent guanines (GG) and occasionally adenines (AG) than DDP at equimolar concentrations, L-OHP-Pt-DNA adducts are more lethal than CDDP-Pt-DNA adducts²⁸. The other reason might be differential DNA repair of L-OHP and DDP lesions. Intact mismatch repair is the main way for DDP induced cytotoxicity; whereas, L-OHP adducts are poorly recognized by mismatch repair proteins^{29–31}.

A number of studies showed that the polymorphisms of TS affect the sensitivity to 5-FU, but the results were controversial. It was discovered that the 3R3R genotype was related with poor outcomes for colorectal cancer patients treated with 5-FU^{32,33}. For GC patients receiving 5-FU regimens, Ott *et al.* also found that 3R3R was a risk factor for the survival³⁴. Whereas, some other studies reported that no significant differences were observed for the survival of patients with different genotypes^{35,36}. Jakobsen *et al.* reported that patients with 3R3R genotype responded better to 5-FU and had longer survival time¹¹. Obviously, the effects of TS 5'-UTR polymorphism on 5-FU treatment are very complicated. The explanation for those controversies may involve in cancer type, genetic background and gene-gene interactions. Our finding was similar to Jakobsen's result (HR = 0.498, 95% CI = 0.259–0.960, $p = 0.032$, Table 2). The 3R allele could enhance TS expression when compared with the 2R allele, and the higher TS expression level caused by 2R3R and 3R3R genotypes would increase the formation of the ternary complex, leading to the improvement of sensitivity to 5-FU. The inhibition of TS may give rise to insufficient dTMP supply in DNA synthesis, dUMP misincorporation into DNA and double-strand breaks followed by cell apoptosis^{37,38}. Controversial data on TS 3'-UTR 6 bp ins > del polymorphism were also reported. In colorectal cancer patients receiving chemotherapy, some studies showed a better outcome of 6bp ins > del polymorphism on the survival^{10,39}, while another report exhibited a higher response rate for patients with TS 6bp ins/ins genotype than those with TS 6bp del/del genotype⁴⁰. Several other studies, similar to our findings, demonstrated that no obvious different survival times were identified among the patients with different genotypes^{41,42}. Interestingly, although no obvious relativity was found between TS 3'-UTR 6bp ins > del polymorphism with patients' survival, the protective effect of TS 5'-UTR 2R > 3R polymorphism was enhanced when chemotherapy patients simultaneous with TS 3'-UTR DI + DD and TS 5'-UTR 2R3R + 3R3R genotypes (HR = 0.332, 95% CI = 0.134–0.822, log-rank $p = 0.046$, Table 5). The interaction between TS 5'-UTR and 3'-UTR may be the explanation for this synergistic effect.

The 677 C > T polymorphism induces an Ala-to-Val switch at codon 222 in MTHFR, resulting in thermolabile variant of the enzyme with reduced activity⁴³. The substitution of Glu-to-Ala at codon 429 in MTHFR caused by 1298 A > C mutation also reduced enzyme activity, but at a lower degree compared to 677 C > T^{43,44}. Although decreased MTHFR activity improve the chemotherapy sensitivity of 5-FU by increasing the level of 5,10-MTHE, the reported data were also inconsistent. For the chemotherapy cohort, some groups reported that MTHFR 1298 A > G and 2756 C > T polymorphism responded negatively to 5-FU for colorectal and gastric cancers patients^{45–47}. Whereas, Omar *et al.* showed that the polymorphism of the MTHFR 677C > T was associated with longer TTP and OS for chemotherapy patients⁴⁸. Some reported that no remarkable relationship was found between MTHFR 1298 A > G and 2756 C > T polymorphisms and patient's survival^{12,49,50}, which was consistent

with our results. The inconsistent results may be caused by diverse schedules, the varied doses of 5-FU used, various cancer stages or types, and various ethnic populations among those studies.

In conclusion, our study systematically investigated the effects of polymorphisms and their interactions of genes in OCM pathway on clinical prognosis of stage II-III GC patients treated with 5-FU-based regimens. Our findings showed that the polymorphisms of MTRR 66A > G, TS 5'-UTR 2R > 3R may serve as potential prognostic markers for GC patients receiving 5-FU-based regimens. Results from this study indicated that gene-gene interactions may enhance or neutralize a drug's anticancer effects, emphasizing that further investigations should be conducted in different cohorts with larger number of patients.

Patients and methods. *Patient samples.* From January 1999 to December 2006, 650 stage II-III patients histopathologically diagnosed with GC were recruited in our study. They underwent surgery at the Yixing People's Hospital (Yixing, Jiangsu Province, China). None of them had received chemotherapy or radiotherapy before operation. 251 patients had 5-FU-based adjuvant chemotherapy, and the other 399 patients had not due to various reasons. The chemotherapy regimens were 5-FU in combination with DDP or L-OHP. Chemotherapy was not conducted until the patients met the following characteristics: platelet counts $\geq 100 \times 10^9/L$, neutrophil counts $\geq 1.5 \times 10^9/L$, hemoglobin level $\geq 8 \text{ g/dl}$ and no sign of organ toxicity. The general clinical characteristics of patients were summarized in Table 1. The median follow-up time is 68.5 months and the maximum follow-up time is 119.0 months. According to Lauren's Standard, the histopathology of GC was classified into intestinal or diffuse types⁵¹. The TNM stages were evaluated according to the TNM classification of the American Joint Committee on Cancer (AJCC cancer staging manual, seventh edition). This study was approved by the Institutional Review Board of Nanjing Medical University (Nanjing, China). And the methods were carried out in accordance with the approved guidelines. All patients enrolled in this study signed an informed consent on using clinical specimen in medical research.

Genotyping. Genomic DNA was extracted from tumor specimens as described previously²³. Polymorphisms of the above genes were determined by SNaPshot technology with either ABI fluorescence-based assay allelic discrimination assay (Applied Biosystems, Forster City, CA) or automated sequencing⁵². The primers used for sequencing in this study are listed in Supplementary Table S4. Genotypes were determined by Genemapper 4.0 software (Applied Biosystems) and the polymorphisms were analyzed in ABI3130 genetic analyzer. About 10% samples were randomly selected to validate genotypes by two people independently. The results were 100% concordant.

Statistical analysis. The overall survival time was calculated from the date of surgery to the last follow up time or death (March, 2009) and mean survival time was chosen if the median survival time (MST) could not be calculated. The relationships of each genotype and combinations of genotypes with clinicopathologic characteristics were elucidated using Student t-test and the Pearson chi-square test according to the types of variables. Log-rank tests and Kaplan–Meier plots were performed by SPSS version 20.0 with two-sided tests and $p < 0.05$ was considered statistically significant (SPSS Inc, Chicago, IL, USA). Univariate or multivariate Cox regression analysis was adopted to estimate adjusted hazard ratios (HRs) and 95% confidence intervals (CIs).

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Author Contributions

J.F.C. and M.-L.H. conceived and designed the experiments. T.T.Z., D.Y.G. and Z.X. performed the experiments. X.Y.H. and P.W. analyzed the data. Y.F.T. and W.D.G. recruited and collected patient information and specimens. T.T.Z. wrote the manuscript. All authors reviewed the manuscript.

Additional Information

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