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Genetic variants in folate metabolism-related genes, serum folate and hepatocellular carcinoma survival: the Guangdong Liver Cancer Cohort study

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Abstract

Folate metabolism is involved in the development and progression of various cancers. We investigated the association of single nucleotide polymorphisms (SNP) in folate-metabolising genes and their interactions with serum folate concentrations with overall survival (OS) and liver cancer-specific survival (LCSS) of newly diagnosed hepatocellular carcinoma (HCC) patients. We detected the genotypes of six SNP in three genes related to folate metabolism: methylenetetrahydrofolate reductase (*MTHFR*), 5-methyltetrahydrofolate-homocysteine methyltransferase reductase (*MTRR*) and 5-methyltetrahydrofolate-homocysteine methyltransferase (*MTR*). Cox proportional hazard models were used to calculate multivariable-adjusted hazard ratios (HR) and 95 % CI. This analysis included 970 HCC patients with genotypes of six SNP, and 864 of them had serum folate measurements. During a median follow-up of 722 d, 393 deaths occurred, with 360 attributed to HCC. In the fully-adjusted models, the *MTRR* rs1801394 polymorphism was significantly associated with OS in additive (per *G* allele: HR = 0·84, 95 % CI: 0·71, 0·99), co-dominant (AG *v.* AA: HR = 0·77; 95 % CI: 0·62, 0·96) and dominant (AG + GG *v.* AA: HR = 0·78; 95 % CI: 0·63, 0·96) models. Carrying increasing numbers of protective alleles was linked to better LCSS ($HR_{10–12\ v.\ 2–6}$ = 0·70; 95 % CI: 0·49, 1·00) and OS ($HR_{10–12\ v.\ 2–6}$ = 0·67; 95 % CI: 0·47, 0·95). Furthermore, we observed significant interactions on both multiplicative and additive scales between serum folate levels and *MTRR* rs1801394 polymorphism. Carrying the variant G allele of the *MTRR* rs1801394 is associated with better HCC prognosis and may enhance the favourable association between higher serum folate levels and improved survival among HCC patients.

Keywords: **Gene polymorphism: methylenetetrahydrofolate reductase: 5-methyltetrahydrofolate-homocysteine methyltransferase: 5-methyltetrahydrofolate-homocysteine methyltransferase reductase: Serum folate: Hepatocellular carcinoma: Survival**

Primary liver cancer (PLC) is the sixth most commonly diagnosed cancer and the third leading cause of cancer death worldwide[(1)]. China alone accounts for over half of the new cases and deaths, with 410 038 new cases and 391 152 deaths in 2020[(1)]. Hepatocellular carcinoma (HCC) is the most predominant type of PLC. The prognosis of HCC is generally poor, with 5-year net survival ranging from 5 to 30 %[(2)]. In addition to the established prognostic factors of underlying liver function, tumour stage,

Abbreviations: GLCC, Guangdong Liver Cancer Cohort; HCC, hepatocellular carcinoma; HR, hazard ratio; LCSS, liver cancer-specific survival; MTHFR, methylenetetrahydrofolate reductase; MTR, 5-methyltetrahydrofolate-homocysteine methyltransferase; MTRR, 5-methyltetrahydrofolate-homocysteine methyltransferase reductase; PLC, primary liver cancer; SNP, single nucleotide polymorphisms; SYSUCC, Sun Yat-sen University Cancer Center.

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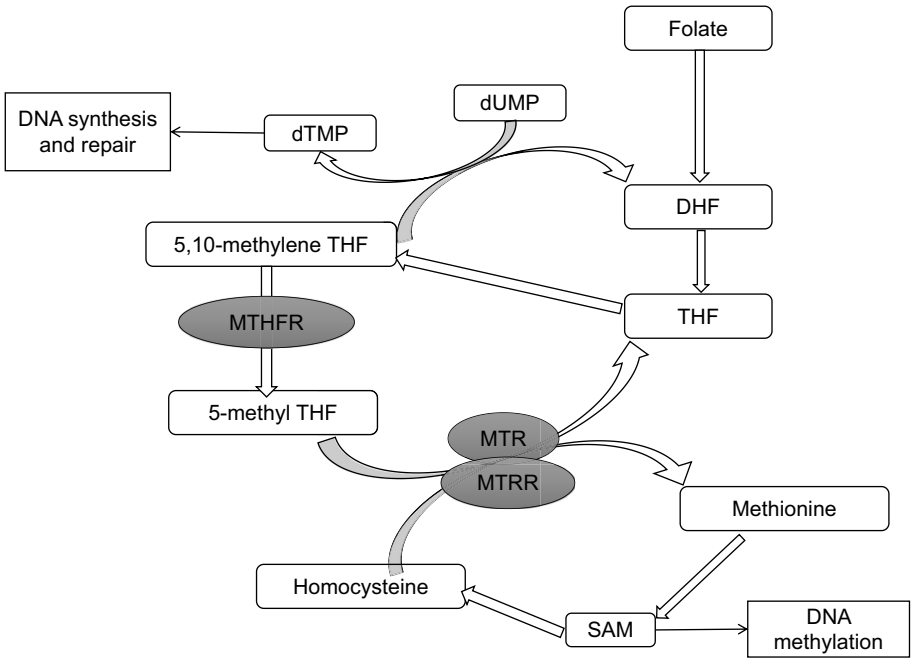

Fig. 1. Overview of folate-mediated one-carbon metabolism (OCM) and related enzymes. 5,10-methylene THF, 5,10methylenetetrahydrofolate; 5-methyl-THF, 5-methyltetrahydrofolate; DHF, dihydrofolate; dTMP, deoxythymidine; MTHFR, methylenetetrahydrofolate reductase; MTR, 5-methyltetrahydrofolate-homocysteine methyltransferase reductase; MTRR, 5-methyltetrahydrofolate-homocysteine methyltransferase reductase; SAM, S-adenosylmethionine; THF, tetrahydrofolate.

performance status and treatments[3], and other factors may also affect HCC prognosis.

Impaired folate-mediated one-carbon metabolism (FOCM) could contribute to cancer development and progression due to the crucial role of folate-mediated one-carbon metabolism in DNA synthesis, repair and methylation[4]. Our previous study has associated lower serum folate concentrations at diagnosis with worse HCC survival[5]. Methylenetetrahydrofolate reductase (MTHFR), 5-methyltetrahydrofolate-homocysteine methyltransferase (MTR) and 5-methyltetrahydrofolate-homocysteine methyltransferase reductase (MTRR) are three key enzymes involved in the folate-mediated one-carbon metabolism (Fig. 1). MTHFR irreversibly catalyses the conversion of 5,10-methylenetetrahydrofolate (5,10-methylene THF) to 5-methyltetrahydrofolate (5-methyl THF), the dominant circulating form of folate. Two common polymorphisms in the *MTHFR* gene, rs1801133 and rs1801131, reduce enzyme activity and lead to lower levels of 5-methyl-THF[6,7]. Previous genome-wide association studies have identified rs1801133 as the gene locus associated with serum folate levels[8]. MTR and MTRR are responsible for the biosynthesis of methionine and the regeneration of THF for nucleotide biosynthesis[9]. Gene variants of *MTR* rs1805087 and *MTRR* rs1801394 may cause decreased activity of the MTR enzyme[10,11]. Previous animal experiments have shown that MTR maintains the tumour tetrahydrofolate pool to drive nucleotide synthesis and cell proliferation in cancer cells[12,13]. Therefore, genetic polymorphisms in the genes encoding folate metabolism-related enzymes may influence enzyme activity and interact with folate status, ultimately affecting cancer survival.

To our knowledge, only six small studies including 71–244 cases have previously investigated the association between several polymorphisms in folate-metabolising genes (*MTHFR* rs1801133, *MTHFR* rs1801131, *MTR* rs1805087 or *MTRR* rs1801394) and the prognosis of HCC and produced inconsistent results[14–19]. Of note, most studies were limited to specific patients, such as patients with chronic hepatitis B-related liver cancer and liver transplant recipients, which may underrepresent the overall patients with HCC. Additionally, most studies solely focused on the individual role of genetic variants in the candidate genes and neglected the potential interaction between these polymorphisms and folate status on the association with HCC survival. To date, only a Taiwanese cohort study suggested that HCC patients carrying the *MTHFR* rs1801133 CC genotype and with high erythrocytes folate levels had worse survival compared with the same genotype[15]. However, apart from the *MTHFR* rs1801133 polymorphism, the role of other genetic variants in folate-metabolising genes and their interactions with folate status in HCC prognosis remain largely unexplored.

Therefore, our aims were (1) to investigate the association between folate-metabolising gene (*MTHFR*, *MTR* or *MTRR*) polymorphisms and the prognosis of HCC and (2) to explore whether these genetic variants modify the association of serum folate concentrations with HCC survival in the Guangdong Liver Cancer Cohort study (GLCC).

Materials and methods

Study population

Our study participants were from the GLCC, an ongoing prospective cohort study initiated in 2013 to investigate factors affecting PLC progression and survival. The study design has been described in detail elsewhere[20]. In brief, we recruited untreated patients aged 18–80 years who were newly diagnosed

with PLC within 1 month at the Sun Yat-sen University Cancer Center, China. A total of 1359 PLC patients were enrolled in the GLCC between September 2013 and April 2017. After excluding fifty-seven cases with a confirmed diagnosis of PLC other than HCC (e.g. intrahepatic cholangiocarcinoma and HCC-ICC) and 332 cases who had no available blood samples for SNP genotyping, a total of 970 eligible patients were included in this study. Among them, 864 patients also had serum folate measurements. The selection of the study participants is presented in online Supplementary Fig. 1.

Ethical approval for this study was obtained from the Ethics Committee of the School of Public Health at Sun Yat-sen University, and the study was conducted according to the Helsinki Declaration of Ethics. All participants provided informed consent at the time of recruitment.

Laboratory assays

Fasting venous blood was collected before anticancer treatment and stored at –80°C after centrifugation. Serum folate concentrations were quantified in batches using a chemiluminescent microparticle immunoassay (ARCHITECT Folate assay, Abbott Diagnostics) at the KingMed Diagnostics Laboratory (Guangzhou, China)[5].

Routine laboratory parameters, including α-fetoprotein, alanine aminotransferase, aspartate aminotransferase, γ-glutamyltransferase, alkaline phosphatase, albumin and total bilirubin and C-reactive protein, were analysed according to a standardised protocol at the Clinical Laboratory of Sun Yat-sen University Cancer Center. We constructed a liver damage score (ranging from 0 to 6) by summing the number of abnormal laboratory-defined values for six hepatic function tests (ALT > 50 μ/l, aspartate aminotransferase > 40 μ/l, γ-glutamyltransferase > 60 μ/l, alkaline phosphatase > 150 μ/l, albumin < 40 g/l and total bilirubin > 20·5 μmol/l) to assess preexisting chronic liver diseases[21]. A score of 0 indicated no liver injury, 1–2 represented possible minor liver injury and ≥ 3 suggested possible liver injury.

Clinical and lifestyle data collection

Demographic characteristics (e.g. age and sex) and diagnostic and treatment information were obtained from the Sun Yat-sen University Cancer Center electronic management system. The Barcelona Clinic Liver Cancer stage was chosen to assess tumour severity, which comprehensively considers tumour number and size, Child-Pugh score (an indicator of the severity of liver dysfunction and hepatic functional reserve)[22] and performance status of the patients[23]. We recorded the primary cancer treatment that patients received after diagnosis.

Information on lifestyles was obtained through baseline interviews using a structured questionnaire. Participants were classified into three groups according to smoking status: never smokers, former smokers and current smokers. Current smokers were defined as those who had smoked at least one cigarette per day for at least 6 months and former smokers were defined as those who had quit smoking for at least 1 year. Weight and height were measured following a standard procedure. BMI was calculated by dividing weight in kilograms (kg) by height in metres squared (m^2).

DNA extraction, genotyping and single nucleotide polymorphisms selection

Genomic DNA was extracted from blood clots using TIANGEN blood clot genomic DNA extraction kits (Tiangen Biochemical Technology (Beijing) Co., Ltd., DP335-02). The purity and concentration of the extracted DNA samples were determined using a Nanodrop one ultra-micro spectrophotometer (Thermo Fisher Scientific), and the ratio of absorbance at 260 and 280 nm (A260/A280) ranged between 1·8 and 2·0 was acceptable. We selected six candidate single nucleotide polymorphisms (SNP) in folate-metabolising genes, including *MTHFR* rs1801133, *MTHFR* rs1801131, *MTHFR* rs2274976, *MTR* rs1805087, *MTRR* rs1801394 and *MTRR* rs10380. We chose SNP with an expected minor allele frequency > 5 % in the Chinese population, which have been previously shown to be associated with HCC or have potential functional significance. These loci were genotyped using the Kompetitive allele specific polymerase chain reaction assay[24]. We designed three primers for each SNP, including two forward-specific primers and one reverse universal primer. Two forward primers corresponded to two kinds of fluorescence signals. After PCR amplification, we measured the fluorescent values of the two signals to determine the genotype of the samples. Strict quality controls were performed by setting up negative and positive controls during the genotyping. The genotype missing rate of *MTHFR* rs1801133, *MTHFR* rs1801131, *MTHFR* rs2274976, *MTR* rs1805087, *MTRR* rs1801394 and *MTRR* rs10380 was 3·4 %, 1·9 %, 1·4 %, 5·8 %, 3·8 % and 3·5 %, respectively.

Survival outcomes

Participants were followed from the date of blood donation until the date of death or the last date known alive whichever occurred first. The last outcome ascertainment was carried out on 22 February 2019. Survival outcomes assessed included overall survival (OS) and liver cancer-specific survival (LCSS). The outcome event was all-cause death for OS and death from HCC for LCSS. The date and cause of death were ascertained by referring to the death registration and reporting system of the Guangdong Provincial Center for Disease Control and Prevention, combined with the inpatient and outpatient medical system of the Sun Yat-sen University Cancer Center. In addition, we conducted telephone-based interviews with the patients or their next-of-kin every 6–12 months to confirm their survival status.

Statistical analysis

We compared clinical and nonclinical characteristics between eligible and ineligible participants, as well as among eligible participants with different genotypes of the selected SNP. Differences among the groups were analysed using one-way ANOVA, Wilcoxon rank sum test or Kruskal–Wallis rank sum test for continuous variables and Pearson's χ^2 test for categorical variables.

Three genetic models (co-dominant (wild-type *v.* heterozygote *v.* homozygote), dominant (wild-type *v.* heterozygote +

homozygote) and additive (per variant allele)) were applied to assess the association between genetic polymorphisms in folate-metabolising genes and LCSS and OS. Cox proportional hazards models were used to calculate hazard ratios (HR) and 95 % CI. Model 1 was a crude model. Model 2 was adjusted for non-clinical factors, including age at diagnosis (continuous), sex (women, men), BMI (< 18·5, 18·5–24·0, 24·0~28·0 and ≥ 28·0 kg/m^2) and smoking status (never, former and current). Model 3 was further adjusted for clinical factors, including α-fetoprotein levels (≤ 400 ng/ml, > 400 ng/ml), C-reactive protein levels (≤ 3·0 mg/l, > 3·0 mg/l), liver damage score (0, 1~2 and ≥ 3), Barcelona Clinic Liver Cancer stage (0, A, B and ≥C) and cancer treatment (hepatectomy/liver transplantation, local ablation, hepatic arterial intervention and other treatments). Since only a few covariates were missing with a small proportion, BMI, C-reactive protein levels and α-fetoprotein levels with missing data (n 3) were automatically excluded from the multivariable models. The proportional hazards assumption was verified using the global Schoenfeld residual test. Sensitivity analyses were conducted by excluding female participants to minimise the impact of gender differences.

Joint associations of folate metabolism-related gene polymorphisms with LCSS and OS were analysed based on the number of protective alleles from the six polymorphisms, where the *C* allele of the rs1801133 polymorphism, the *A* allele of the rs1801131 polymorphism, the *G* allele of the rs2274976 polymorphism, the *A* allele of the rs1805087 polymorphism, the *G* allele of the rs1801394 polymorphism and the *C* allele of the rs10380 polymorphism were considered protective.

We evaluated whether the association between sex-specific quartiles of serum folate concentrations and survival outcomes in HCC patients was modified by the genotype of *MTHFR*, *MTR* and *MTRR*, as well as the number of protective alleles, on both multiplicative and additive scales. The multiplicative interaction was assessed by comparing the −2 log-likelihood of the fully-adjusted models with and without the cross-product interaction term of the sex-specific quartiles of serum folate levels and the SNP genotype (i.e. all tests for interaction are 1 degree of freedom). Stratified analyses by the genotypes were subsequently performed. Linear trends were tested by entering the median value of sex-specific quartiles of serum folate levels as a continuous variable in the regression models. To assess the additive interaction, we treated serum folate concentrations (low-≤median and high->median) and the genotypes (wild-type, mutant) as dichotomised variables, The relative excess risk due to the interaction (RERI) and the attributable proportion due to the interaction (AP) were used to estimate the deviation from the additivity of the effect[25], with the delta method to obtain CI for the indices[26].

Statistical analyses were performed using SPSS version 26·0 (IBM Corp.) and R software version 4·2·3 (R Foundation for Statistical Computing, Vienna, Austria). All P values were two-sided, and $P < 0·05$ was considered statistically significant.

Results

Baseline characteristics

No significant differences in clinical and nonclinical characteristics were observed between eligible and ineligible participants except for cancer treatment received (online Supplementary Table 1). A lower proportion of eligible participants underwent hepatic resection or liver transplantation compared with ineligible participants. Of the 970 patients included, 857 (88·4 %) were men. The mean age at diagnosis was 53·0 (SD 11·9) years. Among the 864 patients who had serum folate measurements, the median serum folate concentration was 6·90 (25th–75th percentile: 5·22–9·20) ng/ml. Nearly half of the patients (46·3 %) were at an advanced stage (i.e. Barcelona Clinic Liver Cancer stage ≥C). Hepatic resection/liver transplantation was the most common tumour treatment (44·0 %), followed by hepatic artery intervention (39·6 %) and local ablation (11·3 %) (Table 1). The distribution of the six selected SNP in the folate metabolism pathway genes is presented in Table 2. The minor allele frequencies of these six SNP ranged between 12·1 % and 28·4 % in HCC patients, which were similar to those in the

Table 1. Baseline characteristics of the included patients with hepatocellular carcinoma in the Guangdong liver cancer cohort study

	Total (n 970)	
Characteristics	*n*	%
Age at diagnosis, years		
mean	53·0	
SD	11·9	
Sex, *n* (%)		
Women	113	11·6
Men	857	88·4
BMI, kg/m^2		
mean	22·77	
SD	3·23	
Smoking status, *n* (%)		
Never smoker	404	41·6
Former smoker	253	26·1
Current smoker	313	32·3
AFP levels, *n* (%)		
≤400 ng/ml	587	60·6
>400 ng/ml	382	39·4
CRP levels, *n* (%)		
≤3·0 mg/l	504	52·0
>3·0 mg/l	465	48·0
Liver damage score*, *n* (%)		
0	208	21·4
1–2	372	38·4
≥3	390	40·2
BCLC stage, *n* (%)		
0	103	10·6
A	303	31·2
B	115	11·9
≥C	449	46·3
Cancer treatment, *n* (%)		
Hepatectomy/liver transplantation	427	44·0
Local ablation	110	11·3
Hepatic arterial intervention	384	39·6
Other treatments†	49	5·1
Serum folate, ng/ml, median (P25, P75)	6·90	5·22, 9·20

Abbreviations: AFP, α-fetoprotein; BCLC, Barcelona Clinic Liver Cancer; BMI, body mass index; CRP, C-reactive protein; P25, the 25th percentile; P75, the 75th percentile, SD: standard deviation.

* A summary score of the number of abnormal laboratory-defined values for six liver function tests: alanine aminotransferase > 50 μ/l, aspartate aminotransferase > 40 μ/l, γ-glutaryl-transferase > 60 μ/l, alkaline phosphatase > 150 μ/l, albumin < 40 g/l and total bilirubin > 20.5 μmol/l, ranging from 0 to 6.

† Including radiation therapy and systemic treatment (e.g. molecular targeted therapy, systemic chemotherapy, traditional Chinese medication).

Table 2. Distribution of the selected SNPs in folate metabolism-related genes in the Guangdong liver cancer cohort study

SNP	Position (GRCh38)	Gene	Allele	Genotype, *n* (%)						MAF (%)	Reference MAF* (%)
				MM		Mm		mm			
			M/m	*n*	%	*n*	%	*n*	%		
rs1801133	1:11796321	MTHFR	C/T	527	56·2	348	37·1	62	6·6	25·2	38·6
rs1801131	1:11794419	MTHFR	A/C	537	56·4	363	38·1	52	5·5	24·5	21·4
rs2274976	1:11790870	MTHFR	G/A	725	75·8	215	22·5	16	1·7	12·3	10·4
rs1805087	1:236885200	MTR	A/G	731	80·0	145	15·9	38	4·2	12·1	10·8
rs1801394	5:7 870 860	MTRR	A/G	468	50·2	400	42·9	65	7·0	28·4	27·3
rs10380	5:7 897 078	MTRR	C/T	637	68·1	280	29·9	19	2·0	17·0	14·6

Abbreviations: M, major allele; m, minor allele; MAF, minor allele frequency; *MTHFR*, methylenetetrahydrofolate reductase; *MTR*, methionine synthase; *MTRR*, methionine synthase reductase; SNP, single nucleotide polymorphism.
* Data were extracted from the National Center for Biotechnology Information (NCBI) Allele Frequency Aggregator (ALFA) for the East Asian population.

general population except for the T allele frequency of *MTHFR* rs1801133 (25·2 % *v.* 38·6 %).

Baseline characteristics of HCC patients by the selected *MTHFR*, *MTR* and *MTRR* genotypes are shown in online Supplementary Tables 2 and 3, respectively. The *MTHFR* rs1801131 CC and *MTHFR* rs2274976 AA genotypes were more prevalent in women than in men. Patients carrying *MTHFR* rs1801133 TT, *MTHFR* rs1801131 CC and *MTRR* rs10380 TT genotypes tended to have lower serum folate levels, whereas patients with *MTHFR* rs2274976 AA, *MTR* rs1805087 GG and *MTRR* rs1801394 GG genotypes tended to have higher serum folate levels compared with their counterparts, although not statistically significant.

Methylenetetrahydrofolate reductase, 5-methyltetrahydrofolate-homocysteine methyltransferase reductase and 5-methyltetrahy hepatocellular carcinoma survival

During a median follow-up of 722 (25th–75th percentile: 320–1077) days, 393 (40·5 %) patients were deceased and 360 (91·6 %) of them died from HCC. The associations of *MTHFR*, *MTR* and *MTRR* genetic polymorphisms with HCC survival are shown in Table 3. After adjustment for non-clinical and clinical prognostic factors, *MTRR* rs1801394 was associated with OS in HCC patients in the additive, co-dominant and dominant models. Compared with HCC patients with wild-type genotype (AA), HCC patients with GA (GA *v.* AA: HR = 0·77; 95 % CI: 0·62, 0·96) genotype exhibited improved OS. In the dominant model, the mutant patients with HCC (AG + GG) had better OS than patients with the wild-type genotype (AA) (HR = 0·78; 95 % CI: 0·62, 0·96). In addition, an increased number of the *G* allele was associated with improved OS (per *G* allele: HR = 0·84; 95 % CI: 0·71, 0·99), under the additive model. However, each additional mutant allele *T* of *MTRR* rs10380 was associated with worse OS among HCC patients (per *T* allele: HR = 1·23; 95 % CI: 1·01, 1·50) under the additive model after full adjustments. The other four SNP (*MTHFR* rs1801133, *MTHFR* rs1801131, *MTHFR* rs2274976 and *MTR* rs1805087) did not show significant associations with the survival outcomes of HCC patients. In sensitivity analyses, after restricting our analyses to male participants, the results were similar to those from the main analyses (online Supplementary Table 4).

When we combined the number of protective alleles, we observed that compared with patients having two to six protective alleles, patients carrying ten to twelve protective alleles had better LCSS (HR = 0·70; 95 % CI: 0·49, 1·00) and OS (HR = 0·67; 95 % CI: 0·47, 0·95) in fully-adjusted models (Table 4).

Interaction between serum folate levels and methylenetetrahydrofolate reductase, 5-methyltetrahydrofolate-homocysteine methyltransferase reductase and 5-methyltetrahydrofolate-homocysteine methyltransferase genetic polymorphisms

In our previous study, patients in the lowest quartile had significantly worse LCSS (HR = 1·48; 95 % CI: 1·05, 2·09) and OS (HR = 1·43; 95 % CI: 1·03, 1·99) after adjustment for non-clinical and clinical prognostic factors compared with those in the third quartile of serum folate[5]. As shown in Table 5, we further observed significant multiplicative interactions between sex-specific quartiles of serum folate concentrations and *MTRR* rs1801394 genotype on the association with LCSS (*P*interaction = 0·016) and OS (*P*interaction = 0·010). When stratified by the genotype of *MTRR* rs1801394, higher serum folate concentrations were associated with better LCSS (Q4 *v.* Q1: HR = 0·55; 95% CI: 0·35, 0·86; *P* = 0·006 for trend) and OS (Q4 *v.* Q1: HR = 0·56; 95% CI: 0·36, 0·86; *P* = 0·006 for trend) among patients who carried mutant genotypes (AG and GG), but not among those with the wild-type genotype (AA). No significant multiplicative interaction was observed between other *MTHFR*, *MTR* and *MTRR* SNPs, as well as the number of protective alleles, and serum folate levels (online Supplementary Table 5).

We documented significant additive interaction between serum folate levels and the *MTRR* rs1801394 genotype on the association with LCSS (RERI = −0·49; 95 % CI: −0·98, −0·003; AP = −0·82; 95 % CI: −1·64, −0·005) and OS (RERI = −0·47; 95 % CI: −0·93, −0·01; AP = −0·80; 95 % CI: −1·58, −0·02). The joint associations of serum folate levels and *MTRR* rs1801394 genotype on survival outcomes of HCC patients are shown in Fig. 2. After adjustment for non-clinical and clinical factors, patients with high serum folate levels (> 6·91 ng/ml) and heterozygous or homozygous variants (AG and GG) had better

Table 3. Association of *MTHFR*, *MTR*, and *MTRR* genetic polymorphisms with survival outcomes among patients with hepatocellular carcinoma in the Guangdong liver cancer cohort study

		Liver cancer-specific survival							Overall survival						
		Deaths/ total	Model 1*		Model 2†		Model 3‡		Deaths/ total	Model 1*		Model 2†		Model 3‡	
Genetic model	Genotype		HR	95 % CI	HR	95 % CI	HR	95 % CI		HR	95 % CI	HR	95 % CI	HR	95 % CI
MTHFR rs1801133															
Co-dominant	CC	193/527	1	ref	1	ref	1	ref	210/527	1	ref	1	ref	1	ref
	CT	133/348	1·05	0·85, 1·31	1·04	0·84, 1·30	1·21	0·96, 1·51	147/348	1·07	0·87, 1·32	1·06	0·86, 1·31	1·21	0·98, 1·51
	TT	20/62	0·81	0·51, 1·28	0·80	0·51, 1·28	1·19	0·74, 1·90	22/62	0·81	0·53, 1·26	0·82	0·52, 1·27	1·12	0·71, 1·77
Dominant	CT + TT *v.* CC	–	1·01	0·82, 1·25	1·00	0·81, 1·24	1·20	0·97, 1·50	–	1·03	0·84, 1·26	1·02	0·83, 1·25	1·20	0·98, 1·48
Additive	Per *T* allele	–	0·97	0·82, 1·15	0·97	0·82, 1·15	1·15	0·96, 1·37	–	0·98	0·84, 1·15	0·98	0·83, 1·15	1·13	0·96, 1·34
MTHFR rs1801131															
Co-dominant	AA	202/537	1	ref	1	ref	1	ref	215/537	1	ref	1	ref	1	ref
	AC	132/363	0·98	0·79, 1·22	1·00	0·80, 1·24	1·01	0·80, 1·26	150/363	1·05	0·85, 1·29	1·07	0·86, 1·31	1·09	0·88, 1·35
	CC	19/52	0·99	0·62, 1·59	1·06	0·66, 1·71	0·91	0·56, 1·49	21/52	1·04	0·66, 1·62	1·11	0·7, 1·74	0·97	0·61, 1·53
Dominant	AC + CC *v.* AA	–	0·98	0·80, 1·21	1·01	0·81, 1·24	0·99	0·80, 1·23	–	1·05	0·86, 1·28	1·07	0·87, 1·31	1·07	0·87, 1·31
Additive	Per *C* allele	–	0·99	0·83, 1·18	1·01	0·85, 1·21	0·98	0·82, 1·17	–	1·03	0·88, 1·22	1·06	0·90, 1·25	1·04	0·88, 1·23
MTHFR rs2274976															
Co-dominant	GG	276/725	1	ref	1	ref	1	ref	297/725	1	ref	1	ref	1	ref
	GA	73/215	0·92	0·71, 1·19	0·95	0·73, 1·23	0·94	0·72, 1·23	85/215	1·00	0·79, 1·27	1·03	0·81, 1·31	1·02	0·80, 1·30
	AA	7/16	1·24	0·58, 2·62	1·24	0·58, 2·65	1·99	0·92, 4·30	7/16	1·16	0·55, 2·45	1·14	0·54, 2·43	1·78	0·82, 3·83
Dominant	GA + AA *v.* GG	–	0·94	0·74, 1·21	0·97	0·76, 1·25	0·99	0·77, 1·27	–	1·01	0·8, 1·28	1·03	0·82, 1·31	1·05	0·83, 1·34
Additive	Per *A* allele	–	0·97	0·78, 1·22	1·00	0·80, 1·25	1·04	0·82, 1·31	–	1·02	0·83, 1·26	1·04	0·84, 1·28	1·08	0·87, 1·35
MTR rs1805087															
Co-dominant	AA	272/731	1	ref	1	ref	1	ref	299/731	1	ref	1	ref	1	ref
	AG	52/145	1·01	0·75, 1·35	1·02	0·76, 1·37	1·12	0·82, 1·51	57/145	1·01	0·76, 1·34	1·02	0·76, 1·35	1·11	0·83, 1·49
	GG	14/38	0·94	0·55, 1·61	0·93	0·54, 1·60	1·29	0·75, 2·24	14/38	0·85	0·50, 1·46	0·87	0·51, 1·49	1·19	0·69, 2·05
Dominant	AG + GG *v.* AA	–	0·99	0·76, 1·30	1·00	0·76, 1·31	1·15	0·87, 1·52	–	0·97	0·75, 1·26	0·98	0·76, 1·27	1·13	0·86, 1·47
Additive	Per *G* allele	–	0·99	0·80, 1·21	0·99	0·80, 1·22	1·13	0·91, 1·40	–	0·96	0·79, 1·17	0·97	0·79, 1·19	1·10	0·89, 1·35
MTRR rs1801394															
Co-dominant	AA	172/468	1	ref	1	ref	1	ref	193/468	1	ref	1	ref	1	ref
	AG	144/400	0·95	0·76, 1·18	0·95	0·76, 1·19	0·82	0·65, 1·03	155/400	0·91	0·74, 1·12	0·92	0·74, 1·13	0·77	0·62, 0·96
	GG	26/65	1·07	0·71, 1·62	1·01	0·67, 1·54	0·88	0·58, 1·34	27/65	0·99	0·66, 1·48	0·93	0·62, 1·39	0·81	0·54, 1·22
Dominant	AG + GG *v.* AA	–	0·97	0·78, 1·19	0·96	0·78, 1·19	0·83	0·67, 1·03	–	0·92	0·75, 1·13	0·92	0·75, 1·13	0·78	0·63, 0·96
Additive	Per *G* allele	–	0·99	0·84, 1·18	0·98	0·83, 1·16	0·88	0·74, 1·05	–	0·95	0·81, 1·12	0·94	0·80, 1·11	0·84	0·71, 0·99
MTRR rs10380															
Co-dominant	CC	228/637	1	ref	1	ref	1	ref	249/637	1	ref	1	ref	1	ref
	CT	112/280	1·13	0·90, 1·42	1·14	0·91, 1·42	1·17	0·93, 1·48	124/280	1·15	0·93, 1·42	1·15	0·93, 1·42	1·20	0·96, 1·49
	TT	8/19	1·30	0·64, 2·64	1·41	0·70, 2·87	2·01	0·97, 4·17	8/19	1·20	0·59, 2·43	1·30	0·64, 2·63	1·83	0·89, 3·78
Dominant	CT + TT *v.* CC	–	1·14	0·92, 1·43	1·15	0·92, 1·44	1·20	0·96, 1·51	–	1·15	0·93, 1·42	1·16	0·94, 1·43	1·22	0·99, 1·52
Additive	Per *T* allele	–	1·14	0·93, 1·39	1·15	0·94, 1·40	1·23	0·99, 1·51	–	1·13	0·94, 1·37	1·15	0·95, 1·39	1·23	1·01, 1·50

Abbreviations: CI, confidence interval; HR, hazard ratio; *MTHFR*, methylenetetrahydrofolate reductase; *MTR*, methionine synthase; *MTRR*, methionine synthase reductase; ref, reference.

* Crude model.

† Adjusted for age at diagnosis (continuous), sex (women, men), BMI (< 18.5, 18.5~24.0, 24.0~28.0, ≥ 28.0 kg/m^2), smoking status (never, former, current).

‡ Additionally adjusted for α-fetoprotein levels (≤ 400 ng/ml, > 400 ng/ml), C-reactive protein levels (≤ 3.0 mg/l, > 3.0 mg/l), liver damage score (0, 1~2, ≥ 3), BCLC stage (0, A, B, ≥C), and cancer treatment (hepatectomy/liver transplantation, local ablation, hepatic arterial intervention, other treatments).

Table 4. Association of combined folate metabolism-related gene polymorphisms with survival outcomes among patients with hepatocellular carcinoma in the Guangdong liver cancer cohort study

No. of protective alleles across six SNPs§	Deaths/Total	HR (95 % CI)					
		Model 1*	95 % CI	Model 2†	95 % CI	Model 3‡	95 % CI
Liver cancer-specific survival							
2–6	45/112	1	ref	1	ref	1	ref
7–8	112/337	0·74	0·53, 1·05	0·75	0·53, 1·06	0·65	0·46, 0·92
9	100/263	0·88	0·62, 1·25	0·84	0·59, 1·20	0·58	0·40, 0·83
10–12	103/258	0·94	0·66, 1·33	0·93	0·65, 1·32	0·70	0·49, 1·00
P for trend‖	–	0·79		0·65		0·017	
Overall survival							
2–6	49/112	1	ref	1	ref	1	ref
7–8	124/337	0·76	0·54, 1·05	0·76	0·55, 1·06	0·66	0·47, 0·92
9	113/263	0·91	0·65, 1·28	0·88	0·63, 1·24	0·60	0·42, 0·84
10–12	107/258	0·89	0·64, 1·25	0·88	0·63, 1·24	0·67	0·47, 0·95
P for trend‖	–	0·63		0·49		0·007	

Abbreviations: CI, confidence interval; HR, hazard ratio; ref, reference.
* Crude model.
† Adjusted for age at diagnosis (continuous), sex (women, men), BMI (< 18.5, 18.5~24.0, 24.0~28.0, ≥ 28.0 kg/m^2), smoking status (never, former, current).
‡ Additionally adjusted for α-fetoprotein levels (≤ 400 ng/ml, > 400 ng/ml), C-reactive protein levels (≤ 3.0 mg/l, > 3.0 mg/l), liver damage score (0, 1~2, ≥ 3), BCLC stage (0, A, B, ≥C), and cancer treatment (hepatectomy/liver transplantation, local ablation, hepatic arterial intervention, other treatments).
§ The *C* allele of the rs1801133 polymorphism, the *A* allele of the rs1801131 polymorphism, the *G* allele of the rs2274976 polymorphism, the *A* allele of the rs1805087 polymorphism, the *G* allele of the rs1801394 polymorphism, and the *C* allele of the rs10380 polymorphism were considered protective.
‖ Linear trend was tested by entering the number of protective alleles as a continuous variable in the regression models.

LCSS (HR = 0·60, 95 % CI: 0·42, 0·85) and OS (HR = 0·59, 95 % CI: 0·42, 0·82), compared with patients with low serum folate levels (≤ 6·91 ng/ml) and the wild-type genotype (AA).

Discussion

We prospectively examined the association of six polymorphisms in three genes encoding folate metabolising enzymes (MTHFR, MTR or MTRR) and their interactions with serum folate concentrations with HCC survival in the GLCC study. Carrying the variant *G* allele of the *MTRR* rs1801394 polymorphism was related to improved OS among patients with HCC after adjusting for potential confounders. However, the *MTHFR* and *MTR* gene polymorphisms did not manifest any association with HCC survival. When combined with the effect of the six SNP effects, patients carrying more protective alleles had better survival. Additionally, we observed multiplicative and additive interactions between serum folate levels and the *MTRR* rs1801394 polymorphism. The association between serum folate concentrations and HCC survival differed by the genotypes of *MTRR* rs1801394 and was only evident among patients who carried mutant genotypes (AG and GG). Compared with patients with low serum folate levels and the wild-type genotype (AA), patients with high serum folate levels and mutant genotypes (AG and GG) had improved LCSS and OS.

MTRR is one of the key regulatory enzymes involved in the folate metabolism pathway. It can catalyse the regeneration of methylcobalamin, which is a cofactor of MTR in the remethylation of homocysteine to methionine, and the regeneration of tetrahydrofolate for nucleotide biosynthesis[27]. Mutations in the *MTRR* gene may decrease the affinity between MTR and MTRR and reduce the activity of MTR, leading to abnormal DNA synthesis, repair and methylation[28]. As well, animal studies have suggested that inhibition of MTR activity suppresses the proliferation of tumour cells[12,13]. rs1801394 is the most common polymorphism in the *MTRR* gene, which causes the substitution of isoleucine with methionine at codon 22 in the MTRR, yielding a variant protein exhibiting fourfold lower enzyme activity than the wild-type protein in vivo[29]. Our results showed that the heterozygous or homozygous mutations (AG and GG) of *MTRR* rs1801394 exhibited a protective effect on the prognosis of HCC. Similarly, AG and GG carriers had a lower risk of recurrence in colorectal adenoma[30] and prostate cancer[31] and better survival of liver cancer[14] and gastric cancer[32,33] than wild-type (AA) patients. The rs10380 polymorphism is also common in the *MTRR* gene. A case–control study conducted in China showed that individuals carrying the *MTRR* rs10380 TT genotype had a higher incidence of HCC than wild-type (CC)[34]. In our study, we additionally observed that each additional variant allele *T* of *MTRR* rs10380 was associated with worse OS in HCC patients. However, no such associations were found in patients with non-small cell lung cancer[35,36] and gastrointestinal cancer[37]. A common variant in *MTR* rs1805087 leads to the substitution of aspartic acid with glycine, which may decrease the activity of MTR. However, our study, along with previous studies on liver[14], lung[35], stomach[32,33] and ovarian[38] cancers, found null associations between the *MTR* rs1805087 polymorphism and cancer survival.

Multiple studies have examined the interactions between the *MTRR* rs1801394 polymorphism and folate intake or status on cancer risk, but there is limited evidence on cancer prognosis. For instance, a case–control study conducted in Thailand found a stronger association between lower serum folate levels and a higher risk of developing colorectal cancer in individuals with the *G* allele (AG and GG) of the *MTRR* rs1801394 than in wild-type individuals[39]. Another case–control study demonstrated that women with wild-type (AA) of *MTRR* rs1801394 and low dietary folate intake have an elevated risk of developing colorectal cancer compared to women with homozygous mutant

Table 5. Association between sex-specific quartiles of serum folate levels and liver cancer-specific and overall survival stratified by *MTHFR*, *MTR* and *MTRR* genetic polymorphisms

		Liver cancer-specific survival				Overall survival			
Genotype	Serum folate levels*	Deaths/total	Adjusted HR†	95 % CI	*P*interaction§	Deaths/total	Adjusted HR†	95 % CI	*P*interaction§
MTHFR rs1801133					0·15				0·16
CC									
	Q1	64/121	1	ref		67/121	1	ref	
	Q2	38/115	0·76	0·50, 1·15		42/115	0·81	0·54, 1·21	
	Q3	28/112	0·59	0·37, 0·93		32/112	0·64	0·41, 0·98	
	Q4	42/115	0·71	0·47, 1·08		46/115	0·73	0·49, 1·09	
	P-trend‡	–	0·08			–	0·10		
CT + TT									
	Q1	35/85	1	ref		38/85	1	ref	
	Q2	38/94	0·94	0·59, 1·51		42/94	0·97	0·62, 1·53	
	Q3	30/98	0·85	0·51, 1·41		35/98	0·91	0·56, 1·47	
	Q4	35/95	0·92	0·57, 1·48		38/95	0·94	0·59, 1·49	
	P-trend‡	–	0·68			–	0·75		
MTHFR rs1801131					0·68				0·70
AA									
	Q1	55/114	1	ref		58/114	1	ref	
	Q2	47/115	0·99	0·66, 1·49		48/115	0·96	0·64, 1·43	
	Q3	35/124	0·67	0·43, 1·04		39/124	0·70	0·46, 1·07	
	Q4	42/116	0·76	0·50, 1·14		45/116	0·77	0·52, 1·16	
	P-trend‡	–	0·09			–	0·13		
AC + CC									
	Q1	46/97	1	ref		49/97	1	ref	
	Q2	30/97	0·68	0·43, 1·10		37/97	0·77	0·50, 1·20	
	Q3	25/87	0·82	0·49, 1·36		30/87	0·89	0·56, 1·44	
	Q4	36/97	0·89	0·56, 1·43		40/97	0·90	0·57, 1·40	
	P-trend‡	–	0·88			–	0·82		
MTHFR rs2274976					0·54				0·70
GG									
	Q1	73/161	1	ref		78/161	1	ref	
	Q2	64/156	1·10	0·78, 1·56		67/156	1·07	0·77, 1·50	
	Q3	48/162	0·79	0·54, 1·15		55/162	0·84	0·59, 1·20	
	Q4	59/165	0·85	0·60, 1·21		63/165	0·85	0·60, 1·20	
	*P*trend‡	–	0·19			–	0·21		
GA + AA									
	Q1	28/53	1	ref		29/53	1	ref	
	Q2	14/55	0·48	0·25, 0·95		19/55	0·63	0·34, 1·17	
	Q3	13/51	0·71	0·35, 1·42		15/51	0·79	0·41, 1·54	
	Q4	19/48	0·69	0·37, 1·28		22/48	0·74	0·41, 1·34	
	*P*trend‡	–	0·45			–	0·50		
MTR rs1805087					0·27				0·39
AA									
	Q1	80/166	1	ref		85/166	1	ref	
	Q2	64/170	0·85	0·61, 1·20		69/170	0·87	0·63, 1·21	
	Q3	46/161	0·67	0·46, 0·97		53/161	0·73	0·51, 1·03	
	Q4	58/160	0·74	0·52, 1·06		65/160	0·78	0·55, 1·09	
	*P*trend‡	–	0·07			–	0·11		
AG + GG									
	Q1	18/41	1	ref		19/41	1	ref	
	Q2	13/37	1·41	0·65, 3·06		16/37	1·47	0·71, 3·04	
	Q3	12/42	1·31	0·58, 2·95		13/42	1·23	0·56, 2·70	
	Q4	14/41	1·61	0·70, 3·69		14/41	1·56	0·69, 3·55	
	*P*trend‡	–	0·30			–	0·36		
MTRR rs1801394					0·016				0·010
AA									
	Q1	41/99	1	ref		44/99	1	ref	
	Q2	39/108	0·88	0·56, 1·38		44/108	0·91	0·59, 1·39	
	Q3	32/108	0·79	0·49, 1·26		38/108	0·84	0·54, 1·32	
	Q4	40/98	1·12	0·72, 1·77		45/98	1·15	0·75, 1·77	
	*P*trend‡	–	0·62			–	0·50		
AG + GG									
	Q1	57/110	1	ref		60/110	1	ref	
	Q2	38/101	0·81	0·53, 1·24		41/101	0·83	0·55, 1·26	
	Q3	25/97	0·62	0·38, 1·00		28/97	0·66	0·42, 1·06	
	Q4	33/108	0·55	0·35, 0·86		35/108	0·56	0·36, 0·86	

Table 5. (*Continued*)

Genotype	Serum folate levels*	Liver cancer-specific survival				Overall survival			
		Deaths/total	Adjusted HR†	95 % CI	*P*interaction§	Deaths/total	Adjusted HR†	95 % CI	*P*interaction§
	*P*trend‡	–	0·006			–	0·006		
MTRR rs10380					0·66				0·75
CC									
	Q1	66/145	1	ref		71/145	1	ref	
	Q2	55/155	0·87	0·60, 1·25		62/155	0·93	0·65, 1·32	
	Q3	37/131	0·79	0·52, 1·19		42/131	0·85	0·57, 1·26	
	Q4	47/136	0·82	0·55, 1·21		51/16	0·83	0·57, 1·22	
	*P*trend‡	–	0·31			–	0·31		
CT + TT									
	Q1	31/63	1	ref		32/63	1	ref	
	Q2	24/59	1·03	0·58, 1·83		25/59	1·02	0·58, 1·78	
	Q3	24/74	0·66	0·38, 1·15		28/74	0·74	0·43, 1·25	
	Q4	29/72	0·78	0·46, 1·33		32/72	0·80	0·48, 1·34	
	*P*trend‡	–	0·21			–	0·27		

HR, hazard ratio; *MTHFR*, methylenetetrahydrofolate reductase; *MTR,* methionine synthase; *MTRR*, methionine synthase reductase; ref, reference; Q1, first quartile; Q2, second quartile; Q3, third quartile; Q4, fourth quartile.

* Sex-specific quartiles of serum folate levels: women: Q1: ≤ 6.65 ng/ml, Q2:6.65~8.97 ng/ml, Q3:8.97~11.00 ng/ml, Q4: > 11.00 ng/ml; men: Q1: ≤ 5.03 ng/ml, Q2:5.03~6.70 ng/ml, Q3:6.70~8.88 ng/ml, Q4: >8.88ng/ml.

† Adjusted for age at diagnosis (continuous), sex (women, men), BMI (< 18.5, 18.5~24.0, 24.0~28.0, ≥ 28.0 kg/m^2), smoking status (never, former, current), α-fetoprotein level (≤ 400 ng/ml, > 400 ng/ml), C-reactive protein level (≤ 3.0 mg/l, > 3.0 mg/l), liver damage score (0, 1~2, ≥3), BCLC stage (0, A, B and ≥C), and cancer treatment (hepatectomy/liver transplantation, local ablation, hepatic arterial intervention and other treatments).

‡ Test the trend against a variable containing the median of each interquartile.

§ The likelihood ratio test was used to evaluate the interaction term.

type (GG) and low dietary folate intake[40]. Similarly, in a cohort of Japanese postmenopausal women, the *MTRR* rs1801394 GG genotype and lower folate intake were associated with a higher risk of breast cancer compared with adequate folate intake and wild-type genotype (AA)[41]. In a randomised controlled trial conducted in 546 patients with colorectal adenoma, the risk of recurrence significantly decreased in *MTRR* rs1801394 heterozygotes and homozygotes (AG and GG) who were treated with folic acid (500 μg/d), but not in those who did not receive folic acid[30]. Consistent with previous results, we observed a significant interaction between the *MTRR* rs1801394 variant and serum folate levels on both additive and multiplicative scales. Compared with the AA genotype carriers with low serum folate levels, individuals carrying the *G* allele (AG and GG) of the *MTRR* rs1801394 and with high serum folate levels (> 6·91 ng/ml) had better HCC survival. The association between higher serum folate levels and improved LCSS/OS was only restricted to patients with the AG and GG genotypes of *MTRR* rs1801394, but not the wild-type (AA) patients with HCC. Our findings suggest that HCC patients with *MTRR* rs1801394 mutations may benefit more from optimising folate status through diet and supplements.

MTHFR is a key enzyme in the folate metabolism pathway that carries out the irreversible conversion of 5,10-methylene THF to 5-methyl THF, which in turn directs the folate pool towards remethylation of homocysteine to methionine. Two common polymorphisms in the *MTHFR* genes, rs1801133 and rs1801131, have been related to the activity of the enzyme MTHFR and altered levels of DNA methylation and synthesis[6,7], but the function of *MTHFR* rs2274976 remains unknown[42]. To the best of our knowledge, the impact of the *MTHFR* rs2274976 polymorphism on HCC prognosis remains unexplored, while the association between *MTHFR* rs1801133 and rs1801131 polymorphisms and HCC survival has yielded inconsistent results. For example, a cohort study reported that HCC patients carrying the *MTHFR* rs1801133 TT and CT genotypes had a higher rate of HCC recurrence compared with those with the CC genotype[18]. However, another cohort study found that *MTHFR* rs1801133 TT and CT genotypes were associated with favourable survival in HCC patients[15], but no association was found between *MTHFR* rs1801131 polymorphism and HCC survival[19]. Previous cohort studies also revealed an interaction between folate intake and *MTHFR* rs1801131 polymorphism on survival among patients with ovarian cancer[43] or esophageal cancer[44,45], where the association between high folate intake and better prognosis was only evident in individuals with the *MTHFR* rs1801133 CC genotype. In contrast, rs1801133, rs1801131 and rs2274976 polymorphisms in the *MTHFR* gene were not associated with survival among patients with HCC in our study. Moreover, the present study did not support that the association between serum folate levels and HCC survival differed by *MTHFR* rs1801133 and the other two *MTHFR* gene polymorphisms (rs1801131 and rs2274976). In agreement with our results, a follow-up study of 232 patients with HCC reported no significant interaction between the *MTHFR* rs1801133 polymorphism and RBC folate levels on survival[15]. The primary circulating form of folate is 5-methyl THF, which is synthesised through the catalytic action of the MTHFR enzyme. The enzyme is responsible for regulating the conversion of folate derived from dietary and/or supplemental sources, thereby *MTHFR* polymorphisms potentially interact with folate intake rather than circulating folate levels, which may explain the discrepancy between studies.

The combined effect of multiple SNP may be more informative than the individual effect of a single SNP. As expected, HCC patients with over seven protective alleles related to folate metabolism had better OS and LCSS compared with patients carrying 2–6 protective alleles. Consistently, in a cohort study of patients with gastric cancer, the combination of the *MTRR* rs1801394 GA and *MTR* rs1805087 AA genotypes

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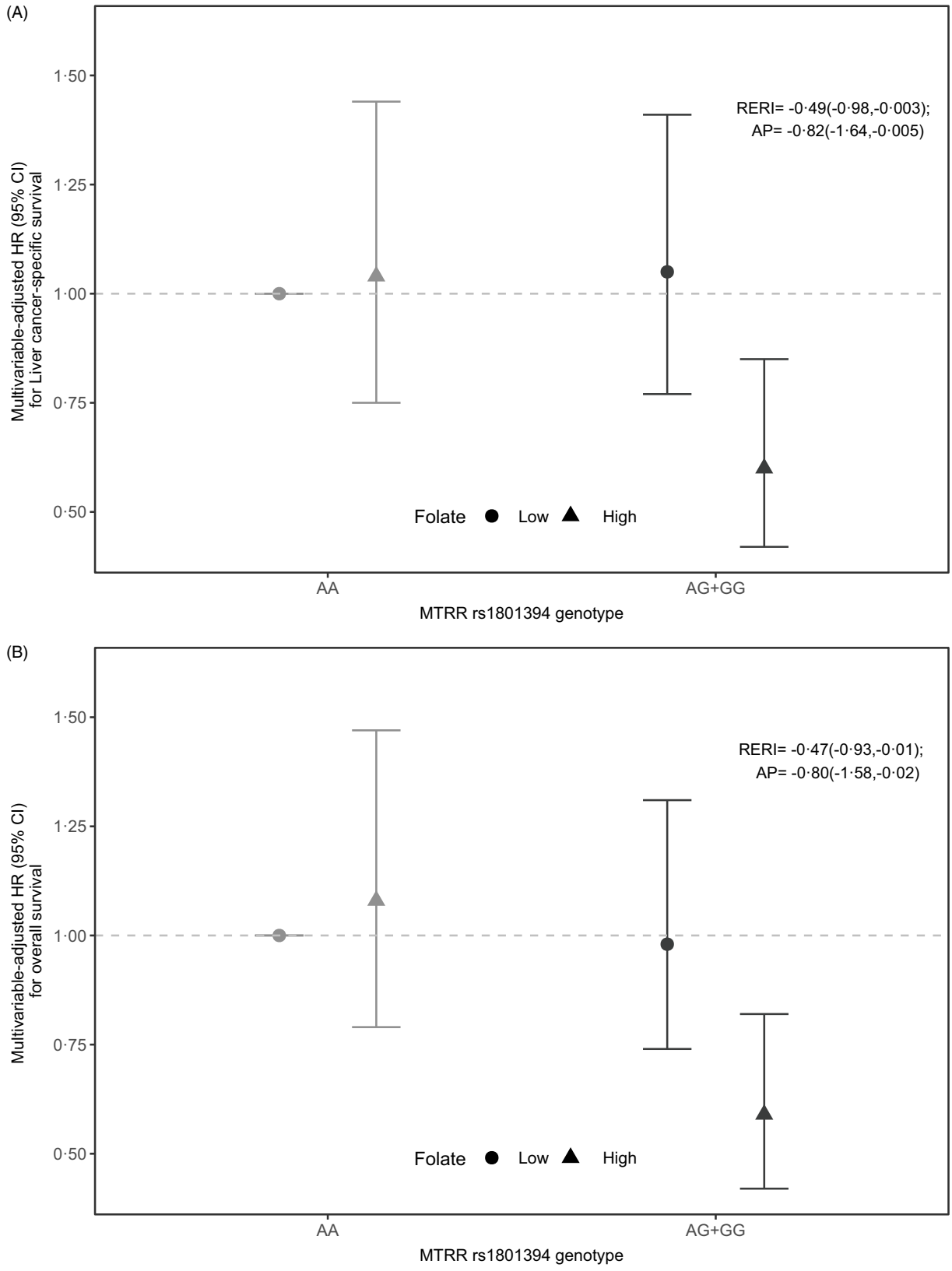

Fig. 2. Joint effects of serum folate levels and genotype of *MTRR* rs1801394 on survival outcomes in the Guangdong Liver Cancer Cohort study. (A) Liver cancer-specific survival and (B) overall survival. AP, attributable proportion due to interaction; HR, hazard ratio; *MTRR*, 5-methyltetrahydrofolate-homocysteine methyltransferase reductase; RERI, relative excess risk due to interaction. The *MTRR* rs1801394 genotype was divided into wild-type (AA) or mutant (AG + GG) groups. Serum folate levels were classified into low (≤ 6·91 ng/ml) and high (> 6·91 ng/ml) based on the median, represented respectively by solid circles and triangles. Data were analysed by Cox proportional hazards models, adjusted for age at diagnosis (continuous), sex (women, men), BMI (<18·5, 18·5~24·0, 24·0~28·0, ≥ 28·0 kg/m^2), smoking status (never, former, current), α-fetoprotein level (≤ 400 ng/ml, > 400 ng/ml), C-reactive protein level (≤ 3·0 mg/l, > 3·0 mg/l), liver damage score (0, 1~2, ≥ 3), BCLC stage (0, A, B, ≥C), and cancer treatment (hepatectomy/liver transplantation, local ablation, hepatic arterial intervention, other treatments). Measures for additive interaction and the corresponding 95 % CIs were estimated using the delta method.

prolonged patient survival over the combination of the *MTRR* rs1801394 AA and *MTR* rs1805087 AA genotypes. However, no significant protective effect was found in patients with the *MTRR* rs1801394 GA and *MTR* rs1805087 GA genotypes[33].

Several strengths of this study lend credibility to its findings. First, we performed a large prospective cohort study and included only patients with newly diagnosed HCC to minimise potential confounding. Second, we genotyped six specific SNP, including SNP (*MTHFR* rs2274976 and *MTRR* rs10380), not previously addressed in prognostic studies of liver cancer. In addition, we measured serum folate levels in the same population, which enabled us to investigate the individual and combined association of folate-metabolising genes (*MTHFR, MTR* or *MTRR*) polymorphisms and serum folate levels with HCC prognosis. Third, we extensively collected information on covariates, including demographics, lifestyle factors, clinical characteristics and cancer treatment. Controlling for these key prognostic factors helps minimise confounding biases. Lastly, we used both OS and LCSS as endpoints in our analyses, despite HCC being a highly lethal cancer. This provides a more comprehensive view of HCC prognosis in our study.

We also acknowledge several limitations in our study. First, we only measured serum folate concentrations at diagnosis of HCC. Changes in diet, lifestyles, cancer progression and treatments such as chemotherapy, which can inhibit the folate cycle, may affect circulating folate levels after diagnosis. Additionally, we did not have information on biomarkers of other one-carbon nutrients such as vitamin B_2, vitamin B_6 and vitamin B_{12}. Moreover, we only detected six common variants in the folate-metabolising genes. However, other genetic variants in these genes could potentially affect the activity of enzymes involved in folate metabolism. Finally, all of our participants were Asian, so it may be challenging to generalise our findings to patients of different genetic backgrounds.

In conclusion, our results showed that the heterozygous or homozygous mutant genotypes of *MTRR* rs1801394, individually or in combination with higher serum folate concentrations, were associated with improved survival among patients with HCC, supporting the role of gene–environment interactions in HCC prognosis. Our study provides the possibility of precision folate intervention to improve the prognosis of HCC with specific folate metabolism genotypes. Future randomised clinical trials and experiments are warranted to confirm our findings and decipher the underlying mechanisms.

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All authors declare no potential conflicts of interest.

The data are available from the corresponding author on reasonable request.

Supplementary material

For supplementary material/s referred to in this article, please visit https://doi.org/10.1017/S0007114524001776

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