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Genetic Polymorphisms of One-carbon Enzymes Interactively Modify Metabolic Folate Stress and Risks of Hepatocellular Carcinoma Development

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Abstract

Few studies have comprehensively evaluated how genetic modifiers of metabolic one-carbon stress as folate deficiency and hyperhomocysteinemia interact to modify the host-susceptibility of human heaptocellular carcinoma (HCC) development. Genetic polymorphisms of 3 key one-carbon enzymes (methylenetetrahydrofolate reductase: MTHFR, methionine synthase: MS, and thymidylate synthase: TS) at 5 loci were characterized in a case-control and hospital-based Asian population (n=398). The monopolymorphic analysis revealed that the *T* variant allele at *MTHFR 677 loci* in relative to the other genotyped variant alleles (*MTHFR 1298C*, *MS 2756G*, *TSER 2R*, *TS3'UTR 1494+6bp* insertion) was associated with a significant 40% reduction of HCC risks in the dominant model (adjusted ORs: 0.6, 95% CI: 0.4-0.9, *P*=0.03). Among individuals with low metabolic folate stress (serum folate>6 ng/mL), the *MTHFR* CC wild-type interacted with the *TSER* 2R variant alleles to increase HCC risks (OR: 0.14 vs. 0.3; 95% CI: 0.1-0.8), whereas 2-fold reduced HCC were associated with the compound *MTHFR T* and *TSER* 2R variant alleles (OR: 0.14 vs. 0.07; 95% CI: 0.02-0.2) (*P* for interaction: 0.044) after adjustment for serum homocysteine (Hcy) levels. The *TSER 2R* or *TS3'UTR+6bp* variant alleles interacted with *MTHFR T* variant allele to reverse its lowering serum folate and elevating Hcy effects (*P* for trend=0.009 and 0.001, respectively). Taken together, our data demonstrated that *MTHFR 677 T* and *TSER* 2R variant allele interacted to alleviate metabolic one-carbon folate stress, which folate-genetic interactions may be the important elements in favor of reduced HCC risks.

Keywords: Metabolic folate stress; Genetic polymorphisms; Hepatocellular carcinoma

Introduction

Serving as one one-carbon donor or acceptor to mediate *de novo* synthesis of purine and pyrimidine, folate-mediated one-carbon metabolism is critical for normal biochemical and physiological function of the liver [1]. Depleted folate status resulted in one-carbon metabolic and genetic stress including elevated homocysteine (Hcy) levels, aberrant DNA methylation, oxidative DNA, lipid and protein damage in hepatocytes and liver tissues [2,3]. Numerous animal studies have demonstrated that elevated one-carbon metabolic stress by dietary folate deprivation led to hepatocellular carcinoma (HCC) development [3-5]. Despite several hepatic disorders subgroups are reported to frequently suffer from folate deficiency [6-8], there are relatively limited human studies to demonstrate relationships between the increased folate-mediated one-carbon stress and HCC carcinogenesis. A prospective high-risk cohort study showed an association of low blood folate with risks for liver damage and HCC on Caucasian population [9]. Asia subjects in high metabolic one-carbon stress as low serum folate and elevated Hcy levels had significantly increased risks for HCC development [10]. The accumulating evidence suggests an universally important role of elevated one-carbon metabolic stress in human HCC development among different races.

Genetic polymorphisms of one-carbon enzymes have been proposed to modulate metabolic one-carbon stress of human subjects. The most studied key enzyme in folate-mediated one-carbon metabolism is the methylenetetrahydrofolate reductase (MTHFR). It irreversibly converts 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate for the remethylation of Hcy to methionine by methionine synthetase (MS), providing the precursor of S-adenosylmethionine (SAM) for DNA methylation [11]. A polymorphism at position 677 (C677T) of the *MTHFR* gene is associated with 30% and 65% reduced enzyme activity in heterozygous (CT) and homozygous (TT) variants, respectively [12]. Another polymorphism at position A1298C of the *MTHFR* gene is associated with moderately reduced the enzyme activity [13]. Individuals with the *MTHFR 677T* or 1298C variant alleles commonly have reduced plasma folate levels and increased Hcy concentrations [14,15]. Reduced MS activity by genetic polymorphism at A2756G was associated with elevated Hcy levels [16].

Thymidylate synthase (TS) is the other rate-limiting enzyme which catalyzes the conversion of dUMP to dTMP for DNA synthesis and repair. The potentially functional *TS* polymorphisms, a 28-bp tandem repeat in the *TS* 5'-untranslated enhanced region (*TSER*) [17], or a 6-bp deletion/insertion at nucleotide 1494 in the 3'-untranslated region of the *TS* gene (*TS 3'-UTR*), were correlated with alteration of *TS* expression [18]. As the TS competes with the MTHFR for their mutual substrate of 5, 10-methylenetetra-hydrofolate in one-carbon metabolism, reduced

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mRNA expression levels affected by *TSER* 2R/2R variant allele or *TS3'UTR* -6/-6 variant alleles [17,19,20] have been associated with lower plasma folate and elevated Hcy levels [18,20-22]. Although the results of the reports are not conclusive, polymorphisms of these one-carbon enzymes not only promoted metabolic stress but also genetic stress such as altered DNA methylation, uracil misincoporation and oxidative DNA damage [23-26], all of which have been proposed as plausible mechanisms in human HCC development.

The full complement of one-carbon enzymes involved in methyl and homocysteine metabolisms is tissue specific. The liver is the major organ with the most active one-carbon metabolism. Hepatic activities of *MTHFR*, *MS* and *TS* could be modulated by availability of their substrate and coenzymes, genetic polymorphisms, rates of one-carbon flux and diseases conditions. Few studies have evaluated how genetic modifiers of metabolic one-carbon stress, mainly emerging from folate deficiency and hyperhomocysteinemia, may interact to modify the host-susceptibility of human HCC development. Several studies reported an increased risk for HCC associated with *MTHFR TT* variant [27,28], whereas others reported a reduced HCC risk for individuals with *TT* genotype [29,30]. The genetic *MS* and *TS* polymorphisms have been individually linked to several cancer risks including colorectal cancers [31-33], yet with relatively little evidence on HCC development. None of the reported results comprehensively evaluates folate-polymorphic interactions on HCC risks. We test the hypothesis in a case-control and hospital-based study on Asian population in the area where a high HCC incident prevailed. DNAs extracted from peripheral blood monocytes (PBMC) of HCC cases and HCC-free controls (n=398) were genotyped for genetic polymorphisms of 3 key one-carbon enzymes at 5 loci. Serum folate and Hcy levels were assayed for metabolic one-carbon stress. Interactions between one-carbon genetic polymorphisms and metabolic folate stress in modifying risks of HCC development were investigated.

Materials and Methods

Study subjects

The present study was carried out in the period Jan 2005 to Aug. 2009. Patients with HCC were recruited from two Medical Centers, Chi-Mei Hospital (CMH) and National Taiwan University Hospital (NTUH) at the Southern and Northern Taiwan, respectively, into the B vitamin and HCC Cancer Prevention Study. Details of recruiting HCC patients with diagnostic criteria for the presence of HCC have been described elsewhere [34]. Briefly, the presence of HCC were diagnosed by two physicians with the data of alpha-fetoprotein elevation (>400 ng/mL), liver imaging (by B-type ultrasonography, computed tomography, magnetic resonance imaging, or/and angiography), and/or histologic exam. In total, 199 HCC patients participated in the entire study. The controls were selected from Community Healthy Exam, who participated in a healthy screening program at Chi-Mei Hospital. The controls were matched by sex with the HCC patients. Exclusion criteria included viral infection, chronic liver diseases, and alcohol abuse as assessed by a medical history, a complete physical examination, and routine laboratory evaluation. The study protocol was approved by the Joint Ethical Committee of Fu-Jen University, Taiwan National University and Chi-Mei Hospital. All participants have provided written informed consent to participate in the study. The written consent content covered (1) IRB preliminary examine research project; (2) The screened eligible control subjects; (3) The clinical trial participants consent, based on regulations of Taiwan National University and Chi-Mei Hospital IRB or Fu-Jen University Ethics Committee.

Blood biochemical determinations

Within 1 week following the diagnosis of HCC presence and prior to the subsequent treatment of HCC in scheduled consultations, patients donated fasting blood samples. Blood of the controls was collected during the time they received health examination. Peripheral blood samples were taken after a 12 h fasting period, chilled, and transported to the laboratory. Plasma and serum samples were immediately separated upon arrival and were stored at -80°C until further analysis. Lymphocytes were purified from whole blood using standard Ficoll-Hypaque centrifugation, and were used for molecular genetic analysis in the study. Folate and total homocysteine (tHcy) levels were measured in the serum samples using commercially available radioimmunoassay kits (Becton Dickinson, Orangeburg, NY), and by fluorescence polarization immunoassay (Becton Dickinson) on an Abbott 130 AxSYM system (Becton Dickinson), respectively.

Genetic polymorphisms

The *MTHFR C677T*, *A1298C*, and *MSA2756G* polymorphisms were determined by real-time PCR and melting curve analysis using a Light Cycler instrument (LightCycler, Roche Diagnostics, Mannheim, Germany). The *TSER* tandem repeat polymorphism and *TS 3'-UTR* polymorphism was analyzed by PCR-RFLP as previously described [17,20]. The forward and reverse primers, the hybridization probes and the amply products for each genotype were presented in Table 1. Briefly, the reaction buffer consisted of 4 μL of Light Cycler DNA Master Hybridization Probe mix (Taq DNA polymerase, reaction buffer, dNTP mixture, and 10 mM $MgCl_2$), 200 nM probe 1, 400 nM probe 2, 200 nM primers, and 50 ng DNA. After a preincubation of the reaction mixture at 95°C for 10 min, thermo cycling was carried out at 95°C for 5 s, 55°C for 5 s, and 72°C for 5 s for 40 cycles. The quality control of each genotype was performed by direct DNA sequencing of separated amplified DNA fragments by electrophoresis in a 2% agarose

Gene	Amplified product	Primer and hybridization probe sequence (5'→3')
MTHFR C677T		
Forward	166 bp	5'-TGG CAG GTT ACC CCA AAG G-3'
Reverse		5'-TGA TGC CCA TGT CGG TGC-3'
Probe 1		5'-TGA GGC TGA CCT GAA GCA CTT GAA GGA GAA GGT GTC T-FL
Probe 2		5'-LC Red640-CGG GAG CCG ATT TCA TCA T
MTHFR A1298C		
Forward	183 bp	5'- CTT TGG GGG AGC TGA AGG ACT ACT AC -3'
Reverse		5'-CAC TTT GTG ACC ATT CCG GTT TG-3'
Sensor [A]		5'-CTT CAA AGA CAC TTT CTT CAC TGG TC 3'-FL
Anchor		5'-640-CTC CTC CCC CCA CAT CTT CAG CAG-3'
MS A2756G		
Forward	290 bp	5'- TTG CTC ATC TAT GGC TAT CTT GCA-3'
Reverse		5'-GAC ACT GAA GAC CTC TGA TTT GAA CTA-3'
Probe 1		5'-GAA GAT ATT AGA CAG GAC CAT TAT G-FL-3'
Probe 2		5'-640-GTC TCT CAA GGT AAG TGG TAG AAA CAG ATT-3'
TSER		
Forward	238 bp or 210 bp	5'-CGT GGC TCC TGC GTT TCC C-3'
Reverse		5'-GAG CCG GCC ACA GGC AT-3'
TS 3'UTR		
Forward	158 bp or 152 bp	5'-CAA ATC TGA GGG AGC TGA GT-3'
Reverse		5'-CAG ATA AGT GGC AGT ACA GA-3'

Table 1: The primers and hybridization probes sequence of nucleotide for the analysis of one-carbon metabolism genes.

gel at 100 V for 40 min. DNA sequencing was conducted with a Big Dye Terminator Cycle Sequencing Kit (Perkin Elmer, Wellesley, MA, USA) and an ABI 377 automated sequencer (Applied Biosystems, Foster City, CA, USA).

Statistical analysis

Statistical analyses were performed using the Statistical Analysis System (SAS/STAT version 6.12, SAS Institute, Cary, NC). Chi-square test was used to examine differences in the distribution of selected demographic variables and in genotypic frequencies between cases and controls. Laboratory data of continuous variables were compared using student t tests between cases and controls. We calculated the geometric mean of levels of serum folate and Hcy levels within strata of *MTHFR* and *TS* genotypes and case-control status using analysis of covariance. Logistic regression models were used to examine the associations between folate status, *MTHFR*, *MS* and *TS* genotypes, and risk of HCC. The strength of a given parameter associated with HCC was measured by its odds ratio and the corresponding 95% confidence interval and 2-sided P value. Dependent variables such as serum folate and Hcy levels that were not normally distributed were log-transformed. Differences were considered to be statistically significant for *P* values of <0.05.

Results

Characterization of metabolic one-carbon stress among the study subjects

As metabolic one-carbon status of the study subjects was profiled using blood biochemical markers, mean serum folate levels of the control and HCC cases were 11.7 ± 6.8 and 7.9 ± 5.2 ng/mL, respectively (Table 2). HCC cases had significantly lower serum folate and elevated tHcy concentrations as compared to the healthy control (*P*<0.001) (Table 2). Metabolic one-carbon stress of the study subjects were characterized by clinical folate deficiency (serum folate <6 ng/mL) and hyperhomocysteinemia (Hcy levels >13 μM). Rates of marginal folate deficiency (43%) and hyperhomocysteinemia (32%) among HCC cases were 3-fold and 5-fold higher than those of the controls (P <0.0001), respectively. The data suggested an elevated metabolic one-carbon stress among HCC cases.

Genetic modifiers of metabolic one-carbon stress in the study subjects

To genotype the subjects, the melting curve-histograms representing for the genetic polymorphisms of *MTHFR C677T, MTHFR A1298C, and MS A2756G* loci of several HCC patients by use of hybridization probe and real-time PCR analysis were shown in Figure 1. Figure 2 shows the representative gel images for the genetic polymorphism of *TSER* and *TS 3'-UTR* using restriction fragment length polymorphism (RFLP) analysis. The quality control of each genotype was confirmed by direct DNA sequencing of amplified DNA products among the designated HCC patients (Figure 3). Genotypic and allelic frequencies of the *MTHFR, MS,* and *TS* polymorphisms at 5 loci among the controls and HCC patients are summarized in Table 3. The data revealed that distribution of *MTHFR C677T* variants was 25% CT and 12% TT in the controls, and 31% CT and 8% TT in the HCC group. The T allelic frequencies marginally differed between the controls and HCC cases (29% vs. 23%, *P*=0.05). Both frequencies of *MTHFR A1298C* variant allele in the control (20%) and cases (19%), and *MS* 2756G variant allele in the control (10%) and cases (10%) were not significantly different. Allelic frequencies for the *TSER 2R* variant allele in the control (17%) and HCC cases (19%) did not significantly differ. Neither did frequencies of the *TS* 3'UTR polymorphisms for the minor allele +6/+6 bp insertion in the control (23%) and the cases (27%). All of the genotype distributions were in agreement with Hardy-Weinberg Equilibrium in both cases and controls (data not shown).

	Control subjects	HCC patients	P values
Age, years	57.4 ± 9.8	62.9 ± 11.4	<0.0001
Sex, male/female	133/66	138/61	0.67
Serum folate, ng/mL	11.7 ± 6.8	7.9 ± 5.2	<0.0001
Deficiency rate[2], n (%)	27 (14)	86 (43)	<0.0001
Homocysteine, μmol/L	9.6 ± 3.7	12.3 ± 5.8	<0.0001
Hyperhomocysteinemia[3], n (%)	11 (6)	63 (32)	<0.0001

Table 2: Base line and metabolic one-carbon stress of the control and HCC patients[1,2]. [[1]Values are expressed as mean ± SD for continuous variables and proportions (%) for categorical variables. Statistical differences were determined by Wilcoxon test for continuous variables and by χ^2 test for categorical variables; [2]Folate and tHcy values were log transformed for statistical tests; [3]Folate deficiency was defined as the serum folate less than 6 ng/mL. [4]Hyperhomocysteinemia is defined as the serum Hcy levels greater than >13 μmol/L].

Single one-carbon polymorphism in relation to HCC risks

Logistic regression analysis for HCC risks in relation to each variant allele of one-carbon polymorphism were examined (Table 4). In relative to the homozygous wild-type C allele at the *677* loci of the *MTHFR* gene, the compound heterozygous and homozygous variant *T* allele were associated with a significant 40% reduction of HCC risks (dominant model: Adjusted ORs: 0.6, 95% CI: 0.4-0.9, *P*=0.03). Conversely, the compound heterozygous or homozygous wild-type C allele had a non-significant 40% increase of HCC risks (recessive model: Adjusted OR: 1.4, 95% CI: 0.7-2.8, *P*=0.4). Neither in the dominant nor the recessive model, *TS* polymorphism at single loci of *TSER* or *TS3'UTR* were not significantly associated with HCC risks. The *MTHFR A1298C* variant allele or the *MS 2756G* variant allele did not correlate with HCC risks (data not shown). For sum of *MTHFR* and *TSER* 2R variant alleles, individuals with 3 or 4 mutant alleles exhibited a 50% reduction in risk of HCC as compared to those with no mutant allele, yet without achieving a statistical significance.

Interactions between the single one-carbon polymorphism and metabolic folate stress on HCC risks

We exam how single loci of each one-carbon polymorphism may interact with metabolic one-carbon stress to modify HCC risks by stratifying serum folate status into high metabolic one-carbon stress (deficient folate level <6 ng/mL) or low stress (normal folate level >6 ng/mL). The data are shown in Figure 4. For those with high metabolic stress in folate-deficient status, the compound *CT* and *TT* variant genotypes as compared with the CC genotype were significantly associated with 80% reduced risks of HCC (OR: 0.2, 95% CI: 0.1-0.6, *P*<0.05) (Figure 1A). For those carrying the homozygous *MTHFR CC* wild-type allele, low metabolic folate stress (normal serum folate >6 ng/mL) was associated with a 90% decrease of HCC risks (OR: 0.1, 95% CI: 0.06-0.3, *P*<0.05). Low metabolic folate stress in combination with T variant alleles (CT+TT genotype) further reduced HCC risks by 20% as compared with the wild-type CC genotype (OR: 0.08, 95% CI: 0.04-0.2, *P*<0.05) (Figure 1A). Genetic polymorphisms of *TS* gene at both 3' and 5'UTR loci did not modify HCC risks among individuals with high metabolic folate stress (serum folate <6 ng/mL) (Figure 1B and C). Regardless of TSER and TS 3'UTR polymorphisms, individuals with low metabolic folate stress (serum folate >6 ng/mL) had a significant 70-80% reduced HCC risks as compared with those in high metabolic folate stress (Figures 1B and 2C). No significant interactions between

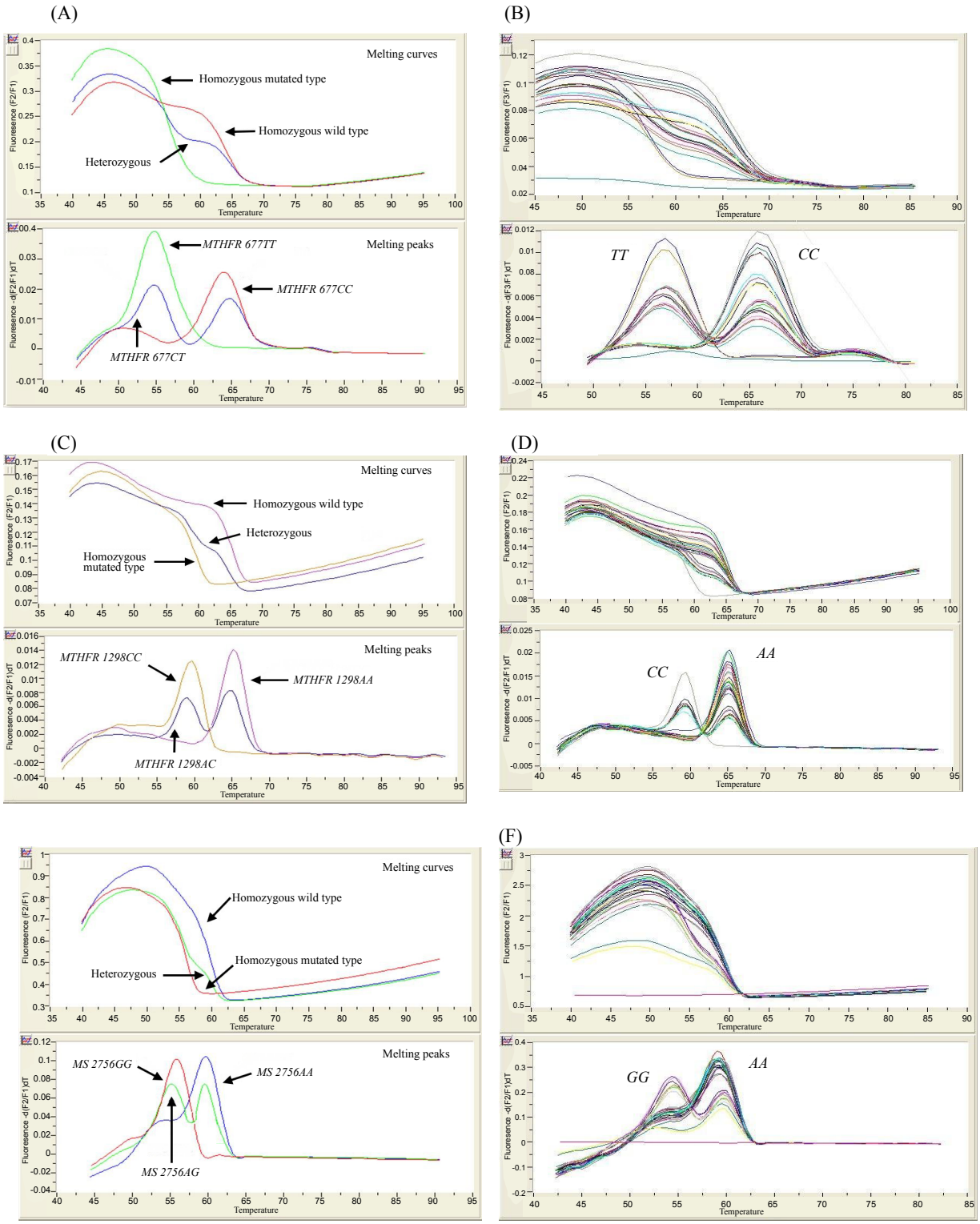

Figure 1: Real-time representative histograms showing genetic polymorphisms at *MTHFR C677T, MTHFR A1298C, and MS A2756G* loci of the subjects. (A) The *MTHFR C677T* genotype was monitored at 640 nm and the melting curve showed a single peak at 64℃ for *CC* samples, a single peak at 55℃ for *TT* samples, and two peaks for heterozygous samples. (B) Genotyping the *MTHFR C677T* polymorphisms among HCC cases (number C24-28) by use of hybridization probe and real-time PCR analysis. (C) The *A1298C* genotype was monitored at 705nm and the melting curve showed a single peak at 63℃ for *AA* samples, a single peak at 60℃ for *CC* samples, and two peaks for heterozygous samples. (D) Genotyping the *MTHFR A1298C* polymorphisms among HCC cases (number C24-28) by use of hybridization probe and real-time PCR analysis. (E) The *MS* A2756G genotype was monitored at 640nm and the melting curve showed a single peak at 60℃ for A/A samples, a single peak at 55℃ for G/G samples, and two peaks for heterozygous samples. (F) Genotyping the MS A2756G *MTHFR* polymorphisms among HCC cases (number C24-28) by use of hybridization probe and real-time PCR analysis.

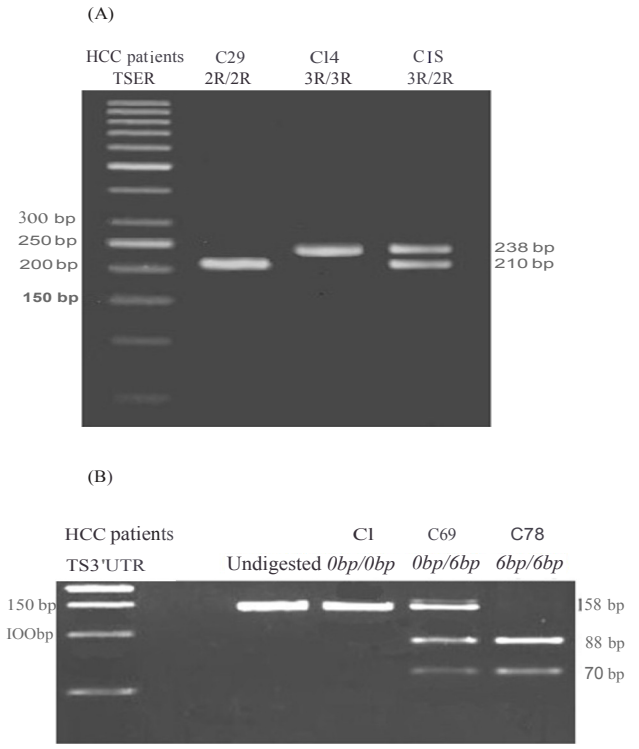

Figure 2: Representative histograms of genetic polymorphism of TSER (A) and TS 3'UTR 1494+6 insertion region (B) using RFLP PCR analysis. (A) Lymphocytic DNAs of the HCC cases (C14, C18 and C29) were assayed for *TSER 3R/3R, 3R/2R, and 2R/2R genotypes.* (B) Lymphocytic DNAs of the HCC cases (C14, C18 and C29) were assayed for *TS 3'UTR 0 bp/0bp, 0 bp/6 bp insertion, and 6 bp /6 bp.*

single one-carbon polymorphism as *MTHFR C677T, 1298C* variant allele, *MS 2756G* variant allele, TS variant allele, and metabolic folate stress in HCC risks were observed (data not shown).

Interactions between the compound MTHFR and the TS genotype, and the metabolic folate stress on HCC risks.

The interactive effects of the compound *MTHFR* and *TS* polymorphisms and metabolic folate stress on HCC risks were assessed (Table 5). As compared with the compound *MTHFR* 677CC and *TS* 3R/3R wild-types (OR: 0.14, 95% CI: 0.05-0.4), two-fold increased HCC risk was associated with the compound *MTHFR* CC allele and *TSER 2R* variant alleles (OR: 0.3; 95% CI: 0.1-0.8), whereas 2-fold reduced risk was associated with the compound *MTHFR T* variant allele and *TSER* 2R variant allele (OR: 0.07; 95% CI: 0.02-0.2). The folate-polymorphic interactions on HCC risks were significant (*P* for interaction: 0.04). No significant polymorphic interaction on HCC risks was found for the compound *TS3'UTR* genotype and *MTHFR CT/TT* genotypes after adjustment for age, gender, and Hcy levels.

Interactions of the single and the compound TS and MTHFR polymorphisms in modifying metabolic one-carbon stress: serum folate and Hcy levels of the study subjects.

We at last investigate how the *MTHFR* C677T or/and *TSER* variant alleles may interact to modify one-carbon metabolic stress of the study subjects, both of which are risk factors for HCC development (Table 6). Among the control subjects, increased numbers of *MTHFR 677 T* variant alleles in relative to the CC wild-type allele were significantly associated with decreasing serum folate (*P* for trend: 0.004) and elevated Hcy levels (*P* for trend: 0.01). No significant effects of the single *TSER or* the *TS3'UTR* polymorphism on serum folate and tHcy levels of the controls were observed. In relative to the homozygous major allele genotypes for *CC-3R/3R as the* reference, the compound *CT-3R/3R genotypes* were significantly associated with reduced serum folate levels (*P*=0.02). The compound *TT-3R/3R genotypes* was associated

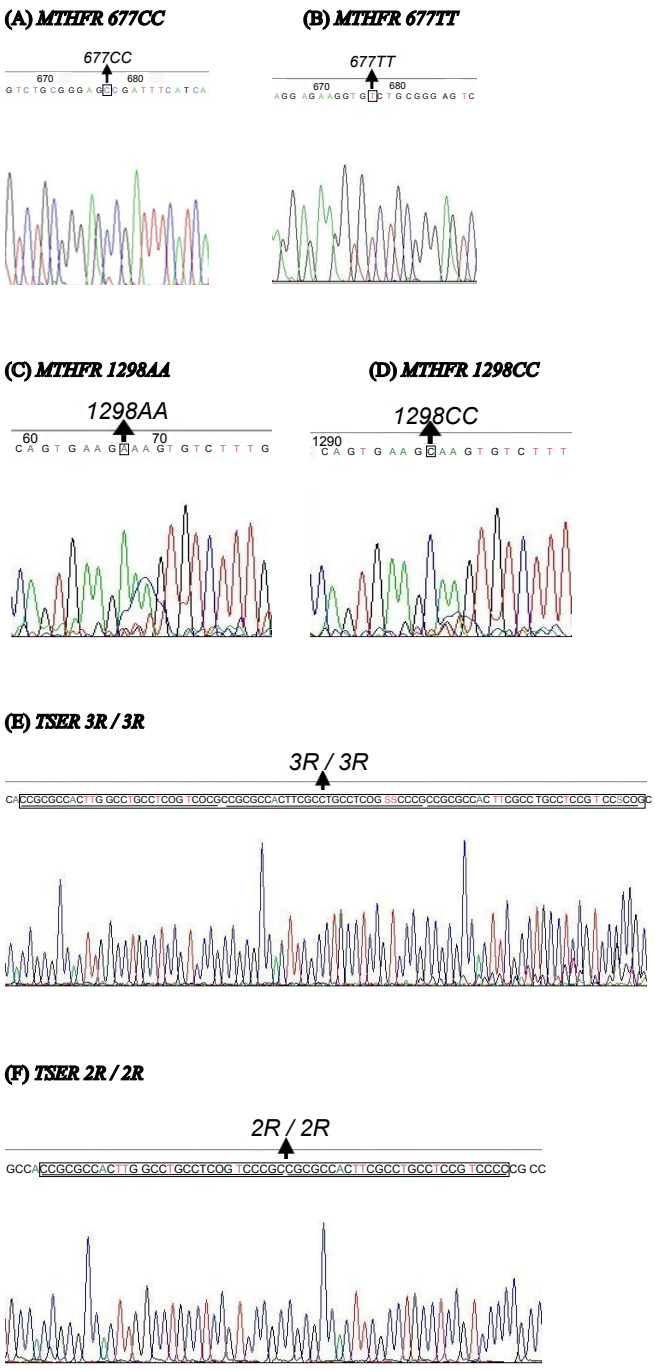

Figure 3: Direct sequence of the *MTHFR gene at C677T and 1298 loci,* and *TSER* gene in lymphocyte DNA of HCC patient. (A) The *MTHFR* 677CC genotype of C14 patients; (B) The *MTHFR 677TT* genotype of C18 patients.; (C) The *MTHFR 1298AA* genotype of C14 patient; (D) The *MTHFR 1298CC genotype of C18 patient*; (E) The *TSER 3R/3R* genotype of C14 patient; (F) The *TSER 2R/2R* of C18 patient.

Genetic polymorphisms	Controls	HCC patients	*P* values
MTHFR C677T, n (%)			
CC	101 (63)	122 (61)	0.11
CT	40 (25)	62 (31)	
TT	19 (12)	15 (8)	
C (%)	71	77	0.05
T (%)	29	23	
MTHFR A1298C, n (%)			
AA	56 (62)	60 (66)	0.53
AC	31 (34)	25 (27)	
CC	3 (3)	5 (5)	
A (%)	79	80	0.84
C (%)	20	19	
MS A2756G, n (%)			
AA	70 (77)	72 (80)	0.72
AG	18 (20)	18 (20)	
GG	2 (2)	0	
A (%)	90	90	1
G (%)	10	10	
TSER, n (%)			
3R/3R	138 (69)	131 (66)	0.71
3R/2R	55 (28)	60 (30)	
2R/2R	6 (3)	8 (4)	
3R (%)	83	81	0.46
2R (%)	17	19	
TS 3'UTR1494, n (%)			
0 bp/0 bp	120 (60)	109 (55)	0.54
0 bp/+6bp	65 (33)	74 (37)	
+6bp/+6bp	14 (7)	16 (8)	
0 bp (%)	77	73	0.29
+6bp (%)	23	27	

Table 3: Genotypic and allelic frequencies of the *MTHFR, MS,* and *TS* polymorphisms at 5 loci among control subjects and patients with hepatocellular carcinoma[1-3] [[1]Abbreviation of the genes: *MTHFR*: Methylenetetrahydrofolate reductase; *MS*: Methionine synthase; TS: Thymidylate synthase; TSER: Enhancer region of TS; TS3'UTR: Un-translate region at 3'end of TS; [2]The 398 subjects were genotyped for *MTHFR C677T*, *TSER* and *TS 3'UTR* polymorphisms. The 180 among 398 subjects were genotyped for *MTHFR A1298C* and *MS A2756G* polymorphisms; [3]Differences in genotypic and allelic frequencies between control subjects and HCC patients were tested by χ^2 test].

with the lowest serum folate levels (P=0.06) and most elevated Hcy levels (P<0.01) as compared with those of the referent. In compound of the variant *T* alleles (CT and TT genotype) with *TSER* 2R variant allele completely negated such *T* allelic effects by normalizing folate and Hcy levels. The compound CC wild-type allele with TSER 2R/2R variant allele, however, reduced serum folate levels as compared with the reference, yet without achieving statistical significance possibly due to the limited subject numbers (n=2).

In relative to the homozygous major allele genotypes of *MTHFR* and *TS3'UTR* for *CC*-0/0bp *as the* reference, the compound *TT*-0/0bp *genotypes* was associated with the lowest serum folate levels (P=0.08) and most elevated Hcy levels (P=0.001). The compound *TT* genotype with heterozygous *TS3'UTR* minor allele genotype (0/+6) negated serum folate lowering effect by T alleles, and in compound with homozygous *TS3'UTR* minor allele genotype (+6/+6) negated Hcy-elevating effects by *T* alleles. Such differentially modifying effects of the *TS* or/and *MTHFR* polymorphisms in serum folate and Hcy levels of the controls were not observed among the HCC cases (data not shown).

Genotypes	Case/Control	OR (95% CI)	P value
*MTHFR C*677T			
CC	223 (122/101)	1.0	-
CT	141 (62/79)	0.7 (0.4~1.0)	0.5
TT	34 (15/19)	0.6 (0.3~1.3)	0.5
P for trend	-	-	0.5
Dominant Model			
CC	223 (122/101)	1.0	-
CT/TT	175 (77/98)	0.6 (0.4~0.9)	0.03
Recessive Model			
TT	34 (15/19)	1.0	-
CC/CT	364 (184/180)	1.4 (0.7~2.8)	0.4
TSER			
3R/3R	269 (131/138)	1.0	-
3R/2R	115 (60/55)	1.2 (0.8~1.9)	0.9
2R/2R	14 (8/6)	1.5 (0.5~4.8)	0.6
P for trend	-	-	0.9
Dominant Model			
3R/3R	269 (131/138)	1.0	-
3R/2R þ 2R/2R	129 (68/61)	1.2 (0.8~1.9)	0.4
Recessive Model			
2R/2R	14 (8/6)	1.0	-
3R/3R + 3R/2R	384 (191/193)	0.7 (0.2~2.2)	0.5
TS3'-UTR			
0/0	229 (109/120)	1.0	-
0/+6bp	139 (74/65)	1.2 (0.8~1.9)	0.7
+6bp/+6bp	30 (16/14)	1.3 (0.6~2.8)	0.7
P for trend	-	-	-
Dominant Model			
0/0	229 (109/120)	1.0	-
0/+6bp + +6bp/+6bp	169 (90/79)	1.2 (0.8~1.9)	0.3
Recessive Model			
+6bp/+6bp	30 (16/14)	1.0	-
0/+6bp + 0/0	368 (183/185)	0.9 (0.4~1.9)	0.7
Sum of *TSER and TS 3'-UTR* variant alleles			
0	187 (91/96)	1.0	-
1	168 (85/83)	1.0 (0.7~1.6)	0.8
2	43 (23/20)	1.2 (0.6~2.5)	0.6
P for trend	-	-	0.8
Sum of *MTHFR* and *TSER* variant alleles			
0-1	310 (160/150)	1.0	-
2	66 (29/37)	0.8 (0.5~1.4)	1.0
3	21 (9/12)	0.5 (0.2~1.4)	1.0
4	1 (1/0)	-	1.0
P for trend	-	-	1.0
Sum of *MTHFR* and *TS 3'-UTR* variant alleles			
0-1	293 (150/143)	1.0	-
2	79 (36/43)	0.8 (0.5~1.3)	1.0
3	23 (12/11)	1.0 (0.4~2.3)	0.7
4	3 (1/2)	0.5 (0.1~5.5)	0.6
P for trend	-	-	0.8

Table 4: Genetic polymorphisms of the methylenetetrahydrofolate reductase (*MTHFR*) and thymidylate synthase (*TS*) genes in relation to risk of hepatocellular carcinoma[1] [[1]Logistical regression analysis with the adjustment for age and sex].

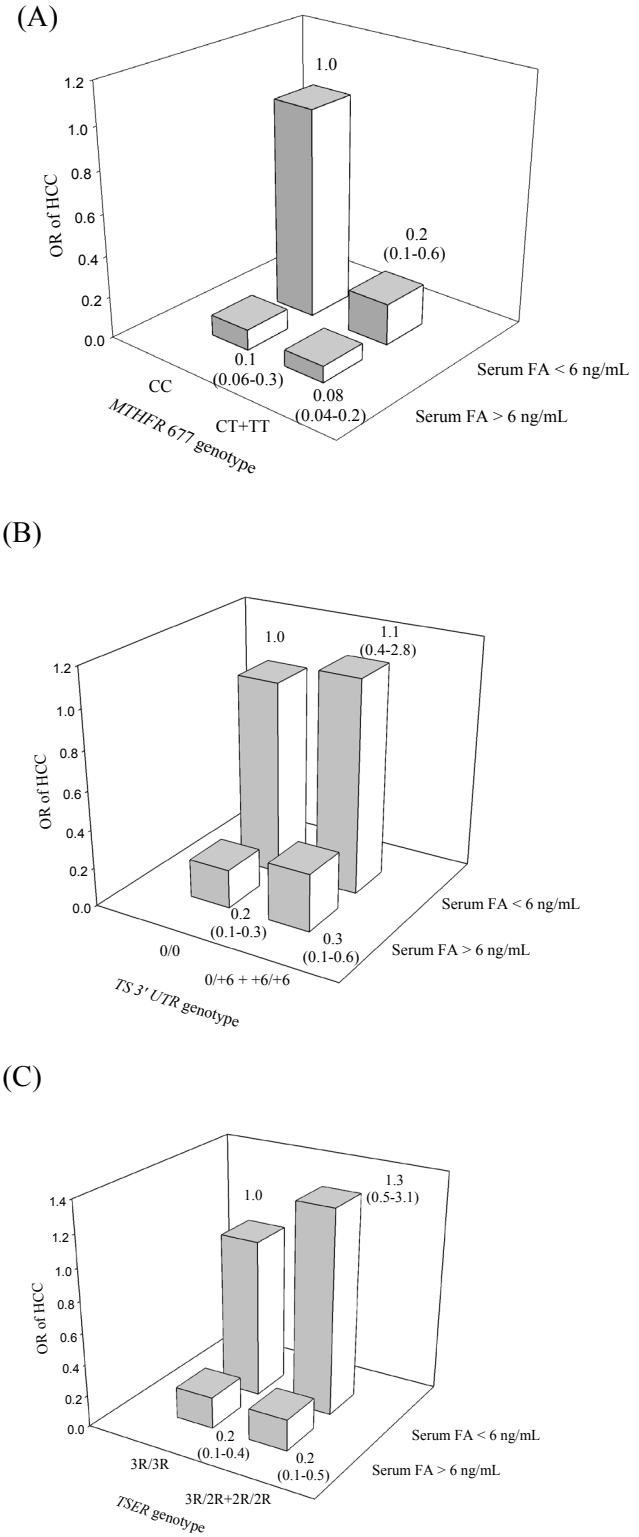

Figure 4: Modifying effects of serum folate levels and genetic polymorphisms on HCC risks. Serum folate levels of the study subjects were stratified into deficient (<6 ng/mL) or normal status (>6 ng/mL), and polymorphic effects on HCC risks on (A) *MTHFR C677T*, (B) *TS* 3'UTR or (C) *TSER* polymorphisms were assessed by logistic regression analysis. HCC risks were adjusted by age, gender and serum Hcy levels [Data were shown by odds ratio (OR) and the corresponding 95% confidence interval (CI) in parenthesis. Statistical significance was set 2-sided P value <0.05 when 95% CI <1.0].

Discussion

Few studies have comprehensively evaluated how genetic modifiers of metabolic one-carbon stress may modify human HCC risks. We found that the *T* variant allele at *MTHFR 677 loci* in relative to the other genotyped variant alleles (*MTHFR 1298C*, *MS 2756G*, *TSER 2R*, *TS3'UTR 1494 +6bp* insertion) was predominantly associated with a significant 40% reduction of HCC risks (ORs: 0.6, 95% CI: 0.4-0.9, *P*=0.03). Based on the mono-polymorphic assessment, several Caucasian population-based studies reported the protective effects of *MTHFR 677TT* alleles against HCC development [29,30,35], whereas the other studies on Asia population did not observe such *T allele*-polymorphic protective effect [27,28]. The possibility for the inconclusive results may in part, if not all, be due to lack of evaluation on the compound polymorphic interactions among the key one-carbon enzymes, corroboration of which activities determined the hepatic one-carbon flow, modulated one-carbon metabolic stress and may interactively modify HCC risks [27-33]. One study by Yuan et al. [30] has demonstrated that the maximum number of mutant alleles in the 3 polymorphic loci of *MTHFR1298, 677, and TS3'UTR* was associated with a significant 62% reduced HCC risks on Caucasians and a non-significant 38% reduced HCC risks on Asians. Similarly, we observed a non-significant 50% reduced HCC risks associated with maximum numbers of variant alleles in the 3 polymorphic loci of *MTHFR677T, TS3'UTR, and TSER* (Table 4). A wide ethnic variation of variant allele frequency reported for *TS* polymorphisms may in part account for the discrepant observations among different races in modifying HCC risks. The *TS 2R variant* allele is less common in Asians (<20%, Table 2) than Caucasians (40%) [36]. The *TS 3'UTR +6/+6* allele was the major allele in Caucasians, whereas this *+6/+6* allele constitutes the minor, and possibly the variant allele genotype in Asians [30,32].

In addition to one-carbon genetic modifiers of HCC risks, metabolic one-carbon stress plays the key role in modulating HCC development in rodents [3-5] and humans [9,10]. The major first-time finding in the present study was to demonstrate that the folate-genetic polymorphisms interacted to modify the host-susceptibility of HCC development. Only among those with low metabolic folate stress, the compound CC genotype and *TSER 2R* variant allele was associated with 2-fold increased HCC risks, whereas the compound *T* and *2R* variant allele was correlated with 2-fold reduced HCC risk (*P* for interaction: 0.044). How folate-genetic interaction may differentially modify HCC risk is unclear. Several mechanisms are plausible. It has been proposed that reduced MTHFR activity by CT and TT genotypes in the absence of other mutations in TS may deviate one carbon flow from remethylation process to de novo thymidylate synthesis, part of which one carbon flow can be redistributed toward purine synthesis with the compound T and 2R variant allele [24]. In particular under normal folate status which provides sufficient one-carbon sources, such one-carbon redistribution by the compound T and 2R variant alleles was reinforced to enrich dNTP pool in favor of DNA repair [25]. Given the fact that the compound *MTHFR T* and *TSER* 2R variant alleles reversed blood folate-lowering and Hcy-elevating effects of TT-allele alone among those with normal blood folate level (Table 6), our observation supported the compound *T* and *2R* variant allelic effects on one-carbon redistribution hypothesis. Enhanced provisions of a better supply of thymidylate and purines by the compound T and 2R variant allele are critical for damaged DNA repair, a defensive mechanism of clearing mutagenic lesions for HCC carcinogenesis [24,31]. It has been reported that the *MTHFR 677TT* genotype was associated with reduced mis-incorporation dU contents of healthy human subjects [23], with reduced DNA mutations of colon tumour [33], and the compound CT/

MTHFR C677T polymorphism	*TS5'-UTR* polymorphism				*TS3'-UTR* polymorphism			
	3R/3R		3R/2R + 2R/2R		0/0		0/+6+6/+6	
	Case/control	OR (95% CI)	Case/control	OR (95% CI)	Case/control	OR (95% CI)	Case/control	OR (95% CI)
Serum folate <6 ng/mL								
677 CC	40 (32/8)	1.0	19 (17/2)	2.3 (0.4~1.3)	36 (30/6)	1.0	24 (20/4)	1.3 (0.3~5.7)
677 CT+TT	25 (16/9)	0.4 (0.1~1.4)	24 (17/7)	0.5 (0.1~1.7)	30 (20/10)	0.4 (0.1~1.3)	20 (13/7)	0.4 (0.1~1.4)
P for interaction	0.54				0.50			
Serum folate ≥ 6 ng/mL								
677 CC	108 (42/66)	0.14 (0.05~0.4)	42 (20/22)	0.3 (0.1~0.8)	85 (32/53)	0.12 (0.04~0.3)	67 (32/35)	0.2 (0.07~0.5)
677 CT+TT	76 (26/50)	0.14 (0.05~0.4)	35 (8/27)	0.07 (0.02~0.2)	64 (18/48)	0.08 (0.03~0.2)	51 (20/31)	0.1 (0.05~0.4)
P for interaction	0.04				0.94			

Table 5: Interactions between MTHFR C677T genotypes, TS genotypes and folate metabolic stress on HCC risk[1,2] [[1]Logistical regression analysis with the adjustment for age, sex and Hcy levels; [2]Folate and tHcy values were log transformed for statistical tests].

Genotype	Folic acid (ng/mL)				Homocysteine (umol/L)			
	n	Mean	SD	*P*	n	Mean	SD	*P*
MTHFR								
CC	101	14.3	9.7	(referent)	101	9.13	2.3	(referent)
CT	79	12.2	7.9	0.10	79	9.06	2.3	0.56
TT	19	10.0	7.5	0.23	19	11.6	4.5	<0.01
P for trend	-	-	-	0.004	-	-	-	0.01
TSER								
3R/3R	138	12.8	8.2	(referent)	138	9.41	2.8	(referent)
3R/2R	55	14.0	10.4	0.06	55	9.11	2.3	0.16
2R/2R	6	11.8	10.5	0.54	6	10.0	2.3	0.80
P for trend	-	-	-	0.30	-	-	-	0.40
TS3'-UTR								
0/0	120	13.1	9.5	(referent)	120	9.27	2.6	(referent)
0/+6	65	12.9	7.5	0.15	65	9.49	3.1	0.62
+6/+6	14	13.9	10.3	0.77	14	9.30	1.5	0.05
P for trend	-	-	-	0.70	-	-	-	0.70
TSER -MTHFR								
3R/3R-CC	76	14.3	9.2	(referent)	76	9.21	2.3	(referent)
3R/2R-CC	23	14.7	11.6	0.40	23	8.87	2.1	0.78
2R/2R-CC	2	10.8	4.3	0.36	2	9.10	0.8	0.22
3R/3R-CT	51	11.4	6.6	0.02	51	9.09	2.5	0.28
3R/2R-CT	24	13.8	9.6	0.91	24	8.75	1.8	0.15
2R/2R-CT	4	12.3	13.3	0.35	4	10.5	2.8	0.45
3R/3R-TT	11	8.25	4.5	0.06	11	12.2	5.2	<0.01
3R/2R-TT	8	12.4	10.1	0.69	8	10.8	3.4	0.73
2R/2R-TT	0	-	-	-	0	-	-	-
TS3'UTR-MTHFR								
0/0-CC	60	14.5	10.6	(referent)	60	9.11	2.0	(referent)
0/+6-CC	33	15.0	8.6	0.44	33	9.04	2.9	0.15
+6/+6-CC	8	10.1	5.5	0.16	8	9.62	1.2	0.06
0/0-CT	50	12.4	8.4	0.23	50	8.95	2.5	0.10
0/+6-CT	25	10.5	4.5	<0.01	25	9.44	2.0	0.84
+6/+6-CT	4	20.9	13.9	0.52	4	8.05	1.6	0.50
0/0-TT	10	8.00	4.8	0.08	10	11.8	4.1	0.001
0/+6-TT	7	11.3	8.4	0.42	7	11.7	5.8	<0.01
+6/+6-TT	2	15.4	16.4	0.46	2	10.5	1.1	0.23

Table 6: Interactions of the TS or/and MTHFR polymorphisms in modifying metabolic one-carbon stress of the control subjects[1,2] [[1]Values are expressed as mean ± SD for continuous variables. Within each genotype or the compound genotypes, the statistical differences between the referent (wild-type genotype) and the variant genotypes were determined by t test at P<0.05. *P* for trend was analyzed by contrast for linear for continuous variables. Differences were considered to be statistically significant at *P*<0.05; [2]Folate and tHcy values were log transformed for statistical tests].

TT genotypes with reduced oxidative DNA damage of lymphocytes of HCC patients [26]. The reported protection against genetic instability due to the reduced MTHFR activity by T allele may serve as the mechanistic basis to confer host-susceptibility of HCC development [37].

Without being in compound with TT genotype, the 2R/2R double repeat in TSER than the triple repeat alone is associated with lower TS expression in HeLaS3 cells [17] or in tumour tissue [19], which may restrict TS activity to induce deregulation of DNA synthesis, repair and cell cycle progression [18,38,39]. The compound *MTHFR* CC wild-type allele with *TSER* 2R variant allele did not enhance substrate provision of 5, 10-methylene-THF to compensate reduced TS activity. By the observation that the compound *MTHFR* CC wild-type and *TSER* 2R variant alleles displayed an adverse effect in folate status (Table 6) and were associated with 2-fold increased HCC risks (Table 6), our data extended to support the differential folate-genetic interaction in one-carbon folate flow in modifying HCC risk.

It should be noted that the significant *T* and *R* allelic interaction of modulating blood folate status and modifying HCC risks was only observed among those with normal folate status, but not for those with folate-deficiency. Similarly, the compound *MTHFR T* and *TS3'UTR* +6/+6bp variant alleles reversed blood folate-lowering and Hcy-elevating effects of TT-allele alone among those with normal blood folate level (Table 6). As the *TS* 3'UTR +6/+6bp alleles in relative to the 0/0bp alleles were associated with higher expression of TS transcripts [20] and higher levels of serum folate levels [18], the enhanced channeling one-carbon flow toward DNA synthesis and repair by such combined variant genotypes of *MTHFR* and *TS* may favor in recycling one-carbon unit to tetrahydrofolate for reentering the one-carbon metabolism and balancing overall one-carbon flux for each metabolic cycle. As modulation of metabolic one-carbon stress by the compound *MTHFR* and TS variant genotypes is only effective under sufficient folate levels of the control subjects, the data suggest a threashold effect of metabolic one-carbon stress for such folate-polymorphic interactions in the protection against HCC progression. In fact, individuals with low metabolic one-carbon stress (serum folate ≥ 6 ng/L) had significantly 80- 90% reduced HCC risks regardless any examined *MTHFR* and *TS* genotype at any tested loci (Figure 4). The beneficial effects of sufficient folate status in supplying one-carbon units for each metabolic cycle of one-carbon metabolism may maximize the favorable one-carbon flux by altered one-carbon enzymatic activity due to different genetic polymorphisms. Our data also provide the plausible explanation why results of the current literature investigating the *MTHFR* or/and TS polymorphic effects in cancer risks are inconsistent [27-29,32,36]. Without consideration of the preexisting one-carbon folate status with the interactive polymorphic impact as the whole, their real functional associations with cancer risks cannot be truly explicated.

Our findings should be interpreted with a few limitations. Due to the unavailability of tumor specimens, analysis of polymorphic-genetic-folate interactions by germ-line genotypes of MTHFR and TS, and serum folate levels may not reflect the true relationships in the target HCC tissues. Small sample sizes may be underpowered for detecting a small but significant association. Larger sample sizes of the studies are needed to clarify whether the TSER or 3'UTR 1494isert6 polymorphism could truly affect one-carbon folate status in the cases and controls. Lastly, the inherent limitations associated with cross-sectional study designs do not depict the causal effect of *MTHFR* and *TS* polymorphisms, folate status and HCC development. Further prospectively designed studies are warranted.

In summary, our data suggest a strong polymorphic effect by reduced MTHFR activity genotype in serum folate and Hcy levels, both in magnitude levels and functional profiles, was differentially influenced by TS variant allelic interaction in normal folate-dependent threshold levels. The compound polymorphic impact of T and 2R variant allele in reduced HCC risk of folate-sufficient individuals supports the hypothesis that deviation of one carbon flux in favor of thymidylate and purine synthesis, possibly for DNA repair, may be a key anticancer mechanism of HCC carcinogenesis. As TS is a target for chemotherapeutic drugs such as 5-fluorouracil, and its mRNA and protein expression levels as the prognostic indicators for certain cancers [40,41], further studies on effects of polymorphic-folate interaction in HCC survival and prognosis are warranted. Since *MTHFR* and *TS* genetic polymorphisms interacted to modulate metabolic stressor of folate status, one-carbon polymorphic identification should be useful to serve as pre-diagnostic markers of metabolic stress in providing alternative strategies in HCC prevention and prognosis.

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Author Disclosures

Chang-Sheng Kuo and Chin-Pao Cheng contribute equally to the study. CS Kuo, CP Cheng, HT Kuo, CH Chen, CY Huang, CC Chen and RFS Huang: No conflicts of interest.

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