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Dietary Risks: Folate, Alcohol and Gene Polymorphisms

Zi-Yuan Zhou[1], Keitaro Matsuo[2], Wen-Chang Wang[3], Huan Yang[1], Kazuo Tajima[2] and Jia Cao[1]

[1]*Department of Hygiene Toxicology , (Key Laboratory of The Ministry of Education of China), College of Preventive Medicine, The Third Military Medical University, ChongQing,*

[2]*Division of Epidemiology and Prevention, Aichi Cancer Center Research Institute, Chikusa-ku, Nagoya,*

[3]*Department of Statistics, College of Preventive Medicine, The Third Military Medical University, ChongQing,*

[1,3]*China*

[2]*Japan*

1. Introduction

Folate, as a member of the water-soluble B-group vitamins, is found widely in foodstuffs. Folate cannot be synthesized by human therefore dietary intake is the only source for human to obtain folate. The pteropolyglumates, usually with 1~6 glutamic acid molecules, are the major forms of natural food folates (Lucock 2000) and the 5-methyl tetrahydrofolate (5-metTHF), derived from hydrolization of absorbed folate and folic acid (the synthetic form of folate) as well, is the primary form in circulation (Ulrich 2005; Pietrzik, Bailey et al. 2010). Through the transmembrane transportation, the 5-metTHF in cell can be reduced by dihydrafolate reductase to tetrahydrofolate (THF) that is directly involved in metabolic process (Lucock 2000; Pietrzik, Bailey et al. 2010), and then performs biological functions in several ways. THF can be metabolized to 5,10-methylene-THF and further be irreversibly reduced into 5-metTHF which is the key step in one-carbon unit metabolism that is catalyzed by the enzyme methylenetetrahydrofolate reductase (MTHFR). By using the methyl donated by 5-methyltetrahydrofolate, the enzyme methionine synthase (MS) converts homocystine to methionine and then the *de novo* synthesized methionine can be catalyzed by the methionine adenosyl transferase to yield S-adenosylmethionine which directly provides methyl for a variety of important *in vivo* methylation reactions (Lucock 2000; Sanderson, Stone et al. 2007). By using 5,10-methylene-THF as methyl donor, the enzyme thymidylate synthase converts deoxyuridylate (dUMP) to deoxythymidylate (dTMP), meanwhile the 5,10-formyltetrahydrofolate from 5,10-methylene-THF is involved in the production of both adenosine and guanosine, all are physiological building blocks of DNA replication (Bollheimer, Buettner et al. 2005; Duthie 2011). Thus, the most prominent function of folate is to transfer and process the one-carbon unit which is needed for methylation reactions and synthesis of thymine and purines. Consequently, folate deficiency

may biologically implicated in physiological processes including base misincorporation and DNA strand breaks, insufficient *de nove* nucleotide synthesis, as well as impaired DNA repair and methylation (Lucock 2000; Ames 2001; Kim 2003; Sanderson, Stone et al. 2007; Duthie 2011).

Therefore, folate has been implicated in colorectal cancer (CRC) because that the steps of folate metabolism may be involved in distinct biological process. A number of epidemiologic and experimental studies concluded that folate may have an inverse association with risk of CRC, however, the results are not consistent and it is argued that too much folate may be unfavourable for preventing the development of CRC especially in those with precursor lesions such as invisible minor adenoma (Giovannucci 2002; Sharp and Little 2004; Strohle, Wolters et al. 2005; Kim 2006; Sanderson, Stone et al. 2007; Sauer, Mason et al. 2009; Kennedy, Stern et al. 2011). The distinct effects of folate on the development of CRC in populations with diversely genetic background suggest that genetic factors, as well as the interaction with folate intake and other coenzymatical factors, may also play a role in the prevention or promotion of colorectal carcinogenesis (Giovannucci 2002; Sharp and Little 2004; Kim 2006; Arasaradnam, Commane et al. 2008; Hubner and Houlston 2009). Growing evidence revealed that the polymorphisms in key folate-metabolism genes may also modify CRC risk in relation to folate intake; but the results are not consistent. Studies suggested that several functional polymorphisms in key genes involved in folate metabolism, such as *MS* A2756G, *MTHFR* C677T and A1298C, may associate with risk of CRC (Giovannucci 2002; Sharp and Little 2004; Kim 2007; Sanderson, Stone et al. 2007; Yu, Zhang et al. 2010). For the *MS* gene, it is still debatable to what extent can the *MS* 2756G variant modulate enzyme activity and plasma homocysteine levels, though evidence from epidemiological studies suggests an association between *MS* A2756G polymorphism and risk of CRC (DeVos, Chanson et al. 2008; Yu, Zhang et al. 2010). To date, several published studies have investigated the role of the *MS* 2756G variant and its interaction with folate intake in the etiology of CRC, mainly in western but few in Chinese populations (Chen, Giovannucci et al. 1998; Ma, Stampfer et al. 1999; Goode, Potter et al. 2004; Ulvik, Vollset et al. 2004; Chen, Jiang et al. 2005; Matsuo, Ito et al. 2005; Ulrich, Curtin et al. 2005; Koushik, Kraft et al. 2006; Theodoratou, Farrington et al. 2008; Yamaji, Iwasaki et al. 2009; Yu, Zhang et al. 2010). For the *MTHFR* gene, the 677T variant encodes an enzyme that is thermolabile, and the heterozygous CT or homozygous TT genotype have nearly 35% or 70% reduction in normal function of the enzyme *in vitro*, respectively (Molloy, Daly et al. 1997). Similarly, the 1298A>C change leads to a decrease in the enzyme activity reportedly *in vitro*, but to a lesser extent, compared with the 677T variant (Weisberg, Jacques et al. 2001). Although the association between *MTHFR* C677T or A1298C polymorphism and CRC risk has also been extensively investigated, the results are not consistent (Chen, Giovannucci et al. 1996; Ma, Stampfer et al. 1997; Chen, Giovannucci et al. 1998; Chen, Ma et al. 2002; Sharp and Little 2004; Ulvik, Vollset et al. 2004; Matsuo, Ito et al. 2005; Huang, Han et al. 2007; Kim 2007).

In addition, alcohol drinking, as one of the known risk factors for CRC, can interfere with the metabolism of folate and one-carbon unit and thus may alter CRC risk in subjects carrying different genotypes. Some earlier studies on folate intake reported that the favourable effects of folate or some genotypes (such as the *MTHFR* 677TT genotype) can be conversely modified by alcohol drinking (Giovannucci 2004; Mason and Choi 2005; Matsuo, Ito et al. 2005). Many, but not all, epidemiological studies further suggested that the interactions among folate intake, alcohol drinking and polymorphisms in genes involved in

folate metabolism are likely presented in the etiology of CRC risk, but the published results were not consistent (Giovannucci 2004; Mason and Choi 2005; Matsuo, Ito et al. 2005; Kim 2007; Kim 2007). Several published studies have investigated the role of folate metabolizing gene polymorphisms and their interactions with folate intake or alcohol drinking in the etiology of CRC, but few in Chinese populations. Therefore, we performed a case-control study to assess the effect of folate intake and some reported functional polymorphisms in genes involved in folate metabolism in the etiology of CRC in Chinese populations, either for their individual effects or the joint effects with alcohol consumption and tobacco smoking.

2. Materials and methods

2.1 Subjects

All cases and controls were recruited from those who were registered into three hospitals of Chongqing City, Southwest China, between January 2001 and September 2004. All cases were newly diagnosed and histopathologically confirmed as having CRC, without any prior cancer history or any chemo-radiotherapies. Controls were cancer-free inpatients from those who had no other severe diseases (i.e., severe cardiovascular diseases, diabetes, severe hypertension, fatty liver and hepatocirrhosis), and without cancer history. Cases and controls were frequency matched by sex, age (±5 years), residence (the same city or county). All subjects were aged between 30-80 years and asked during personal interview to provide a one-time 2~5 ml peripheral blood sample and to complete a questionnaire that elicited information about lifestyles including alcohol drinking and tobacco smoking (1 year prior to the diagnosis for the cases and the time at recruitment for the controls). This study was approved by the Research Ethics Committee of The Third Military Medical University. All subjects provided a signed written informed consent or oral consent if illiterate. Finally, of a total of 1082 cases and 949 controls, we recruited 478 eligible cases (185 colon and 293 rectal cancer) and 838 eligible controls, who had consented to the present study, completed the questionnaires, and provided blood samples.

2.2 Assessment of folate intake and alcohol consumption

Information about folate intake and alcohol consumption one year prior to CRC diagnosis (for cases) or the reference date at recruitment (for controls) was obtained by using the 119-item semi-quantitative food frequency questionnaire developed specifically for Chongqing middle-aged population in our previous work as described elsewhere (Zhou, Takezaki et al. 2004). Briefly, according to the folate content listed in China Food Composition 2002 (Institute of Nutrition and Food Safety. China Center of Disease Control 2002), the frequencies of consumed portion of each food were converted into nutrients; for example, a crude mean of daily folate intake was calculated by multiplying the daily various food intake by its folate content (per 100 grams). The main sources of folate included in the questionnaire were cereals, beans, legumes, nuts, eggs, meats, fishes, bread, edible roots, melons, mushrooms, vegetables and fruits. Similarly, all subjects were also asked to provide detailed information about dietary supplements consumed in the period of one year before diagnosis or recruitment. For alcohol drinking, those who consumed alcohol more than 50 grams each week for more than 6 months were defined as "drinkers". Consumption of all kinds of beverage (beer, alcohol, and wine) was

calculated as pure alcohol volume by their alcohol concentrations (%). For smoking, those who smoked more than four cigarettes each week in average for more than six months were defined as "smokers". Pack-years were calculated by multiplying the total years of smoking by the average packs smoked each day.

2.3 Genotyping

DNA was extracted for each subject from the buffy coat fraction with the Promega DNA Purification Wizard kit (Promega Co. Madison, WI). Genotyping was performed by the polymerase chain reaction restriction-fragments-length polymorphism analysis according to methods described previously for *MS* A2756G (De Marco, Calevo et al. 2002), *MTHFR* C677T (Chen, Giovannucci et al. 1998) and A1298C (Yi, Pogribny et al. 2002), respectively. For the *MS* A2756G polymorphism, a 285-bp PCR product was digested with HaeIII at 37°C and visualized after electrophoresis; the genotypes identified were: 265bp for AA, 265bp, 185bp, 80bp for AG, and 185bp, 80bp for GG. For *MTHFR* C677T polymorphism, a 198-bp PCR product was digested with Hinf I at 37°C and visualized after electrophoresis; the genotypes identified as follows: 198bp for CC, 198bp, 175bp, 23bp for CT, and 175bp, 23bp for TT. For *MTHFR* A1298C polymorphism, a 128-bp PCR product was digested with MboII at 37°C and visualized after electrophoresis; the genotypes identified were: 72bp, 28bp, 28bp for AA, 100bp, 72bp, 28bp for AC, and 100bp, 28bp for CC. The 23bp and 28bp fragments had been electrophoresed out of the gel and cannot be seen. Two cases and four controls failed to be amplified for *MTHFR* and *MS* polymorphisms, possibly due to poor quality of DNA. For genotyping quality control, electrophoresis results of genotypes were identified by a double-blind check and tested for Hardy-Weinberg equilibrium. Furthermore, randomly selected 92 cases and 136 controls were re-genotyped, and there were no discrepancies between the original and repeated genotyping results.

2.4 Statistical analysis

Energy-adjusted daily folate intake was categorized into quartiles based on the distribution in the controls, odds ratios (ORs) and their 95% confidence intervals (CI) were calculated by unconditional logistic regression models to estimate the strength of association between CRC risk and folate intake, the lowest quartile was used as the reference in OR calculation and was further adjusted for sex, age (in years), family cancer history of first degree relatives (yes vs. no) and second degree relatives (yes vs. no). Alcohol consumption status was divided into four categories based on alcohol (g/d) consumed: non-drinker (0), <30 g/d, 30~100 g/d, and >100 g/d for estimation of ORs (95% CI), and the drinking status was only classified into non-drinker and drinker when exploring the interaction with genotypes. Similarly, smoking status was also divided into four categories based on pack years smoked: non-smoker (0), ~10, 10~20, and >20 pack years for estimation of ORs (95% CI), and the tobacco exposure status was only classified into non-smoker and smoker when exploring the interaction with genotypes.

For evaluating the association between CRC risk and polymorphisms of *MS* (A2756G) and *MTHFR* (C677T and A1298C) genes, both ORs (95% CI) with and without adjustment for sex, age, family cancer history of first and second degree relatives, BMI of 10 years ago (divided into 4 subgroups: <20, 20~22.5, 22.5~25.0, >25.0), alcohol drinking and smoking status (yes vs. no) were calculated by using unconditional logistic regression models. ORs (95% CIs) for gene-gene or gene-environment interactions were assessed on a multiplicative

scale in the unconditional logistic regression model with and without adjustment for sex, age, family cancer history of first and second degree relatives. When analyzing the joint effects of two polymorphisms, we used the combined common genotypes as the reference. Hardy-Weinberg equilibrium in the controls was checked for all genotyping data with the χ test, and the exact *P* value was used to assess any departure of genotypes.
All statistical analyses were performed by using SAS (version 8.0, SAS institute, Cary, NC), with two-sided tests and a significance level of 0.05.

3. Results

3.1 Baseline characteristics

Table 1 shows the characteristics of 478 cases and 838 controls included in the final analysis. Overall, the cases were slightly older than the controls with a mean age of 54.3 years for cases and 52.0 years for controls. There was no difference in the distributions of sex, education, and BMI. However, these differences were further adjusted for their residual effects in the later analyses.

Variables	Cases (%) N=478	Controls (%) N=838
Age		
Mean	54.3±12.4	52.0±11.3
≤50 Years	171 (35.8)	358 (42.7)
51-60 Years	137 (28.7)	287 (34.3)
.≥61 Years	170 (35.4)	193 (23.0)
Gender		
Male	273 (57.1)	462 (55.1)
Female	205 (42.9)	376 (44.9)
Educations		
Illiterate	51 (10.7)	75 (8.9)
Primary, middle school	287 (60.0)	526 (62.8)
High school	98 (20.5)	151 (18.0)
College or upper	42 (8.8)	86 (10.3)
BMI (10 years ago)		
Mean	21.6±2.4	21.6±2.5
< 20.0	121 (25.3)	216 (25.8)
20-22.5	200 (41.8)	345 (41.2)
22.5-25.0	113 (23.6)	207 (24.7)
> 25.0	44 (9.2)	70 (8.4)

Table 1. Characteristics of cases and controls in the present study

3.2 Folate intake, alcohol consumption, genotypes, and CRC risk

The results of multiple logistic regression analyses of risk of CRC and folate intake, alcohol consumption, and tobacco smoking are summarized in Table 2.

Environmental Factor	Total Cases/ Controls	Total OR[a] (95%CI)	Male Cases/ Controls	Male OR[a] (95%CI)	Female Cases/ Controls	Female OR[a] (95%CI)
Folate intake (mean, μg/d)						
Lowest. (324.0)	188/209	1.00	85/92	1.00	103/117	1.00
Lower (442.3)	103/210	0.55 (0.40-0.76)	56/112	0.47 (0.30-0.76)	47/98	0.60 (0.38-0.95)
Middle (522.8)	100/210	0.53 (0.38-0.75)	64/116	0.52 (0.32-0.84)	36/94	0.52 (0.31-0.86)
Highest (673.7)	87/209	0.46 (0.31-0.67)	68/142	0.45 (0.28-0.74)	19/67	0.41 (0.21-0.82)
P_{trend}=		0.004		0.005		0.002
Alcohol drinking						
Non-Drinkers	305/612	1.00	116/249	1.00	189/363	1.00
Light ~30 g/d	75/90	1.99 (1.38-2.88)	60/78	1.93 (1.26-2.95)	15/12	2.69 (1.20-6.04)
Moderate ~100 g/d	62/89	1.64 (1.10-2.44)	62/88	1.62 (1.07-2.45)	0/1	NA
Heavy >100 g/d	36/47	1.98 (1.21-3.25)	35/47	1.95 (1.17-3.24)	1/0	NA
P_{trend}=		0.001		0.002		0.011
Years of alcohol drinking						
~5 years	314/624	1.00	123/258	1.00	191/366	1.00
6~20 years	48/55	2.35 (1.50-3.69)	41/49	2.30 (1.38-3.81)	7/6	3.04 (0.96-9.57)
20~ years	116/159	1.67 (1.20-2.31)	109/155	1.58 (1.11-2.23)	7/4	3.85 (1.07-13.81)
Ptrend=		0.001		0.005		0.009
Smoking						
Non-Smoker	280/521	1.00	77/156	1.00	203/365	1.00
Light ~10PY	42/78	1.20 (0.75-1.91)	41/71	1.45 (0.88-2.41)	1/7	0.28 (0.03-2.34)
Moderate 10~20PY	52/75	1.37 (0.87-2.15)	51/73	1.54 (0.96-2.47)	1/2	0.77 (0.07-8.91)
Heavy >20PY	104/164	1.26 (0.87-1.83)	104/162	1.40 (0.95-2.07)	0/2	NA
P_{trend}=		0.187		0.087		0.169

[a] Adjusted for age, gender, cancer history of first and second degree relatives and total energy intake.

Table 2. Odds Ratios (95% CI) of CRC associated with folate intake, alcohol consumption and smoking

In the four groups categorized by the quartile of folate intake, the CRC risk decreased significantly with the increasing folate intake in a dose-response manner (the same trend kept significance in either males or females). Compared with the lowest quartile (a mean intake of 324.1 μg/d), the ORs (95% CI) for the lower (442.3 μg/d), middle (522.8 μg/d) and

highest (673.7 μg/d) folate intake levels were 0.55 (0.40-0.76), 0.53 (0.38-0.75) and 0.46 (0.31-0.67), respectively (P_{trend} = 0.004), and the same trend kept significance in either males or females as described in table 2. Alcohol users had significantly increased CRC risks by nearly 1.6-2.0 folds higher than those who never drank. The ORs (95% CI) for those who consumed alcohol <30g/d, 30-100g/d, and >100g/d were 1.99 (1.38-2.88), 1.64 (1.10-2.44) and 1.98 (1.21-3.25), respectively (P_{trend}=0.001). A longer drinking time was associated with significant increased CRC risk (P_{trend}=0.001). Compared with those who drank less than five years, those who drank 6-20 years or more than 20 years had higher CRC risks with ORs (95% CI) of 2.35 (1.50-3.69) and 1.67 (1.20-2.31), respectively. However, we did not observe statistical evidence of an association between smoking and CRC risk in this study population.

Table 3 describes the distributions of *MS* A2756G, *MTHFR* C677T and A1298C polymorphisms and their associations with CRC risk. Those rare *MS* 2756G genotypes carriers had a 1.5 fold increased risk (OR=1.49; 95% CI: 1.11-1.96; P_{trend}=0.017), and the same trend was observed in the rare homozygous GG carriers though without significant difference. However, Both the C677T and A1298C polymorphisms of the *MTHFR* gene were not associated with CRC risk in this study population. We also tested the joint effect or locus-locus interaction. By using the combination of any two common genotypes as the reference, there were no meaningful interactions between *MS* A2756G and *MTHFR* C677T / A1298C polymorphisms.

Genotypes	Cases (%)	Controls (%)	OR[a] (95%CI)	OR[b] (95%CI)
MS A2756G				
AA	359 (75.4)	688 (82.4)	1.00	1.00
AG	113 (23.8)	141 (16.9)	1.52 (1.15-2.01)	1.50 (1.13-1.99)
GG	4 (0.8)	6 (0.7)	1.06 (0.29-3.88)	1.12 (0.30-4.11)
AG+GG	117(24.6)	147 (17.6)	1.50 (1.13-1.99)	1.49 (1.11-1.96)
P_{trend}=			0.009	0.017
MTHFR C677T				
CC	202 (42.3)	349 (41.9)	1.00	1.00
CT	210 (43.9)	362 (43.4)	0.97 (0.76-1.25)	0.99 (0.77-1.27)
TT	66 (13.8)	123 (14.7)	0.90 (0.63-1.28)	0.87 (0.61-1.24)
CT+TT	276 (57.7)	485 (58.2)	0.96 (0.76-1.21)	0.96 (0.76-1.22)
P_{trend}=			0.684	0.609
MTHFR A1298C				
AA	295 (62.0)	512 (61.3)	1.00	1.00
AC	158 (33.2)	282 (33.8)	0.96 (0.75-1.22)	0.96 (0.75-1.22)
CC	23 (4.8)	41 (4.9)	0.95 (0.55-1.62)	0.96 (0.56-1.65)
AC+CC	181 (38.0)	323 (38.7)	0.95 (0.75-1.21)	0.96 (0.76-1.21)
P_{trend}=			0.634	0.766

[a] Adjusted for age, gender, cancer history of first and second degree relatives and total energy intake; [b] Adjusted for age, gender, cancer history of first and second degree relatives, total energy intake, BMI of 10 years ago, alcohol drinking and smoking

Table 3. CRC risk associated with genotypes of *MS* and *MTHFR* gene

We further evaluated whether the impaction of folate intake on CRC risk was modulated by *MS* and *MTFHR* genes, alcohol drinking or tobacco smoking as summarized in Table 4.

Interactions	Cases/ Controls	OR[a] (95% CI)	Cases/ Controls	OR[a] (95% CI)	$P_{interaction}$
Folate × *MS* 2756	2756AA		AG+GG		
Lowest	145/179	Ref.	42/28	1.84 (1.07-3.16)	
Lower	74/168	0.55 (0.38-0.79)	29/42	0.84 (0.49-1.45)	
Higher	78/177	0.54 (0.37-0.79)	21/32	0.83 (0.45-1.53)	
Highest	62/164	0.46 (0.30-0.71)	25/45	0.65 (0.37-1.16)	0.556
Folate × *MTHFR 677*	677CC		CT+TT		
Lowest	77/80	Ref.	111/127	0.93 (0.62-1.41)	
Lower	44/88	0.55 (0.33-0.90)	59/122	0.50 (0.32-0.79)	
Higher	36/91	0.43 (0.25-0.72)	64/117	0.58 (0.36-0.92)	
Highest	45/90	0.54 (0.32-1.02)	42/119	0.35 (0.21-0.60)	0.629
Folate × *MTHFR 1298*	1298AA		AC+CC		
Lowest	111/138	Ref.	76/69	1.34 (0.88-2.05)	
Lower	66/120	0.69 (0.46-1.04)	37/90	0.50 (0.31-0.81)	
Higher	61/124	0.63 (0.41-0.97)	38/85	0.53 (0.32-0.87)	
Highest	57/130	0.54 (0.34-0.85)	30/79	0.47 (0.27-0.80)	0.205
Folate × Drinking	Non-Drinkers		Drinkers		
Lowest	134/163	Ref.	54/46	1.63 (1.00-2.65)	
Lower	72/154	0.58 (0.40-0.84)	31/56	0.80 (0.46-1.38)	
Higher	58/159	0.46 (0.31-0.69)	42/51	1.17 (0.69-1.99)	
Highest	41/136	0.39 (0.24-0.62)	46/73	0.86 (0.52-1.44)	0.188

Interactions	Cases/ Controls	OR[a] (95% CI)	Cases/ Controls	OR[a] (95% CI)	$P_{interaction}$
Folate × Smoking	Non-Smokers		Smokers		
Lowest	124/144	Ref.	64/65	1.06 (0.65-1.73)	
Lower	67/134	0.57 (0.38-0.85)	36/76	0.55 (0.32-0.96)	
Higher	52/131	0.45 (0.30-0.70)	48/79	0.72 (0.42-1.21)	
Highest	37/112	0.39 (0.24-0.65)	50/97	0.59 (0.35-1.01)	0.208

[a] Adjusted for age, gender, cancer history of first and second degree relatives, total energy intake and also adjusted for smoking status or drinking status, whenever appropriate.

Table 4. CRC risk associated with interactions between folate intake and genotypes (*MS* A2756G and *MTHFR* C677T and A1298C) or environmental factors (alcohol and smoke)

Foalte intake was inversely associated in a dose-dependent manner with CRC risk independent of the three genotypes of *MS* A2756G, *MTHFR* C677T and A1298C. Results in groups stratified by each genotype (common or rare) were similar, though the protective effect of folate intake almost disappeared in those carrying rare *MS* 2756 AG or GG genotype; there was no evidence of an interaction between folate intake and each polymorphism in a multiplicative model. When non-drinkers having the lowest level of folate intake was used as the reference, however, folate intake (from lower to highest) was associated with significantly decreasing CRC risk among non-drinkers (OR=0.39, 95% CI: 0.24-0.62 for the highest level), whereas the significance of protective effect of folate intake shown in Table 2 almost disappeared among drinkers who even had the highest level of folate intake (OR=0.86, 95% CI: 0.52-1.44); similarly, the protective effect of folate intake varied in smokers. Though alcohol drinking or tobacco smoking appeared to have an attenuated protective effect of folate intake, we failed to observe a statistically significant interaction with either drinking ($P_{interaction}$=0.188) or smoking ($P_{interaction}$=0.208) (Table 4).

3.3 Gene-environment interaction

Results of further analyses stratified by alcohol and smoking status are shown in Table 5. Here, we did observe a statistically significant interaction between the *MS* A2756G polymorphism and alcohol intake. An increased risk of CRC was observed in those alcohol drinkers carrying AG or GG genotype, whereas no significant association with alcohol drinking was observed among those carrying the AA genotype ($P_{interaction}$=0.04); Compared with non-drinkers carrying *MS* 2756 AA, the ORs (95% CI) for AG or GG genotype carriers who were drinkers of light (~30 g/d), moderate (30~100 g/d) and highest (~100 g/d) level were 2.84 (1.44-5.60), 3.14 (1.44-6.83) and 4.40 (1.88-10.32), respectively. We also observed a borderline significant interaction between *MTHFR* A1298C polymorphism and alcohol intake ($P_{interaction}$=0.07). For the 1298 AA genotype carriers, the CRC risk of drinkers was 2.0-

Interactions	Case/ Control	OR[a] (95% CI)	Case/ Control	OR[a] (95% CI)	$P_{interaction}$
MS 2756	2756AA		AG+GG		
MS 2756×Drinking					
Non-Drinker	242/502	1.00	62/107	1.19 (0.83-1.71)	
Light ~30 g/d	52/73	1.83 (1.20-2.78)	22/17	2.84 (1.44-5.60)	
Moderate ~100 g/d	44/76	1.43 (0.92-2.24)	18/13	3.14 (1.44-6.83)	
Heavy >100 g/d	21/37	1.46 (0.81-2.63)	15/10	4.40 (1.88-10.32)	0.041
MS 2756×Smoking					
Non-Smoker	224/427	1.00	55/91	1.13 (0.77-1.65)	
Light ~10PY	32/62	1.16 (0.70-1.95)	10/16	1.40 (0.60-3.28)	
Moderate 10~20PY	37/61	1.25 (0.76-2.06)	15/14	1.95 (0.87-4.35)	
Heavy >20PY	66/138	0.96 (0.64-1.45)	37/26	2.90 (1.61-5.22)	0.006
MTHFR 677	677CC		CT+TT		
MTHFR 677×Drinking					
Non-Drinker	133/250	1.00	172/358	0.89 (0.671.18)	
Light ~30 g/d	29/47	1.39 (0.81-2.39)	46/43	2.32 (1.42-3.82)	
Moderate ~100 g/d	25/32	1.80 (0.97-3.31)	37/57	1.36 (0.82-2.25)	
Heavy >100 g/d	15/20	1.83 (0.87-3.82)	21/27	1.82 (0.95-3.47)	0.780
MTHFR 677×Smoking					
Non-Smoker	130/214	1.00	150/304	0.81 (0.60-1.09)	
Light ~10PY	18/36	0.96 (0.50-1.83)	24/42	1.14 (0.62-2.09)	
Moderate 10~20PY	16/39	0.78 (0.39-1.54)	36/36	1.63 (0.92-2.89)	
Heavy >20PY	38/60	1.17 (0.69-1.98)	66/103	1.10 (0.70-1.72)	0.268
MTHFR 1298	1298AA		AC+CC		
MTHFR 1298×Drinking					
Non-Drinker	179/375	1.00	125/234	1.14 (0.85-1.52)	
Light ~30 g/d	46/50	2.40 (1.51-3.81)	28/40	1.66 (0.85-2.88)	
Moderate ~100 g/d	46/59	1.97 (1.23-3.14)	16/30	1.22 (0.61-2.44)	
Heavy >100 g/d	24/28	2.39 (1.30-4.42)	12/19	1.64 (0.75-3.57)	0.069
MTHFR 1298×Smoking					
Non-Smoker	165/323	1.00	114/195	1.09 (0.81-1.49)	
Light ~10PY	27/42	1.50 (0.85-2.66)	15/36	0.92 (0.46-1.82)	
Moderate 10~20PY	34/45	1.46 (0.84-2.51)	18/30	1.32 (0.67-2.60)	
Heavy >20PY	39/102	1.38 (0.89-2.13)	34/62	1.12 (0.66-1.89)	0.377

[a] Adjusted for age, cancer history of first and second degree relatives and total energy intake, smoking status or drinking status, whenever appropriate.

Table 5. CRC risk associated with interactions between genotypes (*MS* A2756G and *MTHFR* C677T and A1298C) and environmental factors (drinking and smoking)

2.4 folds of non-drinkers; however, for those carrying 1298 AC or CC genotype, alcohol drinking was non-significantly associated with CRC risk, compared with non-drinkers carrying the 1298 AA genotype (Table 5).

We also tested the gene-smoking interaction. The patterns of risk associated with *MS* A2756G genotypes seemed to vary by smoking status. For example, smoking was found to be associated with significantly increased CRC risk in *MS* 2756G carriers but not in 2756AA carriers, and there was evidence of an interaction ($P_{interaction}$=0.006). Compared with non-smokers carrying the AA genotype, an OR of 2.90 (1.61-5.22) was observed for those with AG or GG genotype and the highest smoking level (>20 pack-years). However, there were no evidence of an interaction between the genotypes of *MTHFR* C677T or A1298C and smoking (Table 5).

4. Discussion

4.1 Folate intake, alcohol drinking, *MS* A2756G polymorphism and CRC risk

Folate is traditionally regarded as a protective factor for CRC, and many studies have reported a beneficial role in reducing CRC risk, especially in some large-scale case-control or cohort studies (Giovannucci 2002; Terry, Jain et al. 2002; Sanjoaquin, Allen et al. 2005; Strohle, Wolters et al. 2005; Kennedy, Stern et al. 2011), but in recent years some clinical intervention trials have raised the controversy that an increased CRC risk may be produced when folate, especially fortified or supplemental folic acid (synthetic), was administered in an excessive dose and was inopportunely administered when there has some existing lesions (such as undetectable small cancer or precursors) (Strohle, Wolters et al. 2005; Hubner and Houlston 2009; Sauer, Mason et al. 2009). Nonetheless, there is no confirmative evidence against the hypothesis that the loss of homeostasis of folate-mediated one-carbon metabolism can cause abnormal DNA methylation or DNA misincorporation, thus resulting in colorectal neoplasia, but some studies argued that the folate (natural or synthetic) per se can indeed contribute to the reduction of CRC risk (Bollheimer, Buettner et al. 2005; Strohle, Wolters et al. 2005; Kim 2007; Sauer, Mason et al. 2009). The present study investigated the association between folate intake and the risk of CRC in a Chinese population, in which no one had the habit of daily use of any vitamin supplement; therefore, the "folate intake" evaluated in this study means only from natural food, and our results showed a significant association between higher folate intake and lower CRC risk (Table 2). Such a protective effect did not change substantially before and after multivariate adjustment, even in subgroups of colon or rectal cancer (data not shown). Therefore, the present study, generally consistent with many previously published reports (Giovannucci 2002; Terry, Jain et al. 2002; Sanjoaquin, Allen et al. 2005; Kennedy, Stern et al. 2011), provides a further insight and a support for an inverse association between folate (from food) intake and CRC risk in Chinese populations.

Although folate intake alone showed a significant protection against CRC risk, the variation in *MS* and *MTHFR* genes may also play important roles in the folate-mediated methyl cycles, and both alcohol drinking and cigarette smoking are known to impair the absorption and biological actions of folate. Although there was no evidence for an interaction in the present study, there was a trend that the protective effect of the folate appeared to be more obvious in those who were not exposed to the known risk factors drinking or smoking) compared with those who were exposed; in fact, the significant inverse association between folate intake and CRC risk was observed in 2756AA carriers or non-drinkers (non-smokers)

but not in either 2756G carriers or drinkers (or smokers). It seems that, to some extent, the favourable effect of folate may be impaired by the *MS* 2756G allele or drinking (or smoking), a finding consistent with other published studies (Kim 2007; Kim 2007). However, because our study was relatively small, larger studies, especially in Chinese populations, are needed to validate such an interaction between folate intake and *MS* 2756 AG+GG genotypes or drinking (smoking).

4.2 Gene polymorphisms and CRC risk

Several studies have investigated the association between the *MS* 2756 A>G polymorphism and CRC risk but generated conflicting results (Chen, Giovannucci et al. 1998; Ma, Stampfer et al. 1999; Goode, Potter et al. 2004; Ulvik, Vollset et al. 2004; Matsuo, Ito et al. 2005; Ulrich, Curtin et al. 2005; Koushik, Kraft et al. 2006; Theodoratou, Farrington et al. 2008; Yamaji, Iwasaki et al. 2009; Yu, Zhang et al. 2010). Using the common AA genotype as the reference, four studies reported no overall effect but a non-significantly association between CRC risk and the 2756G genotypes (Koushik, Kraft et al. 2006; Theodoratou, Farrington et al. 2008; Yamaji, Iwasaki et al. 2009). Recently, a Japanese study and an American study with 257/771 and 513/609 cases/controls, respectively, also supported the trend that the *MS* 2756G genotypes can elevate the risk of colorectal cancer or adenomas (Goode, Potter et al. 2004; Matsuo, Ito et al. 2005). One population-based case-control study of colon, but not rectal, cancer found no association (Ulrich, Curtin et al. 2005); only two earlier studies (one cancer and one adenomas) found significantly reduced risk among AG or GG carriers (Chen, Giovannucci et al. 1998; Ma, Stampfer et al. 1999), and one large-scale nested case-control study reported an inverse association between the G allele and CRC risk in Norwegians (Ulvik, Vollset et al. 2004). The present study was the first to explore the effect of the *MS* polymorphism on CRC risk in a Chinese population, and we found that the *MS* 2756 AG or GG genotypes were significantly associated with increased risk of CRC, further supporting a positive association between rare 2756 AG or GG genotypes and CRC risk.

We also observed another interesting finding that the frequency of the *MS* 2756G allele (9.2%) among controls was materially different from those (15%~20%) among different ethnic populations reported by other studies, and the frequency of 2756GG genotype in our study was less than 1% in both cases and controls, much lower than 3%-5% in other populations, such as Americans, Europeans and other Asia populations of Japanese or Hindoo (Goode, Potter et al. 2004; Ulvik, Vollset et al. 2004; Chen, Jiang et al. 2005; Matsuo, Ito et al. 2005; Ulrich, Curtin et al. 2005; Koushik, Kraft et al. 2006; Diwakar, Rudresh Kumar et al. 2008; Theodoratou, Farrington et al. 2008; Yamaji, Iwasaki et al. 2009). However, our results, especially for the frequencies of AG and GG genotypes among controls, were very similar to other studies in Chinese populations that investigated the association between the *MS* A2756G polymorphism and Alzheimer disease or lung cancer, in which the G allele frequency for controls was 8.5% and 9.8%, respectively (Liu, Jin et al. 2008; Zhao, Li et al. 2008). Therefore, it is likely that the allele frequency of the 2756G in Chinese is quite different from that of western or other Asia populations such as Japanese or Hindoo. It is still unclear whether the *MS* 2756A>G polymorphism has any functional consequences in its enzyme activity, but it was suggested that this polymorphism may probably decrease the enzyme activity, since the polymorphic site lies in a region connecting the vitamin B_{12} binding domain and the activation domain (Matthews, Sheppard et al. 1998). Considering epidemiologic evidence and the rare 2756G allele frequency in Chinese populations, our

findings suggested this variant may play a role in the etiology of CRC in Chinese populations, possibly a risk factor of CRC for Asia populations, in contrast to a protective effect in other ethnic populations. However, this finding needs to be further validated in larger studies of Asia populations.

In the present study we found that *MTHFR* 677 or 1298 variants were non-significantly associated with decreased CRC risk which is consistent in trend with other earlier epidemiological studies (Chen, Giovannucci et al. 1996; Ulvik, Vollset et al. 2004; Matsuo, Ito et al. 2005; Huang, Han et al. 2007; Kim 2007). The frequencies of 677T or 1298C alleles in our controls were very close to those of other Chinese populations and different Asia populations, such as Japanese or Korean, although the 1298C allele frequency was a slightly lower compared with western white populations (Chen, Jiang et al. 2005; Matsuo, Ito et al. 2005; Kim 2007). Laboratory evidence suggested that the rare 677T or 1298C allele can result in decreased enzyme activity *in vitro* (Molloy, Daly et al. 1997; Weisberg, Jacques et al. 2001) which seemed to favor an increased CRC risk, but on the contrary, most reported studies have not found an significant association between these *MTHFR* polymorphisms and CRC risk, and some earlier studies even reported an inverse association especially in white populations that were likely to have a relatively higher average total folate intake, partly due to use of vitamin supplements (Chen, Giovannucci et al. 1996; Ma, Stampfer et al. 1997). One-carbon unit metabolism may depend on a series of enzymatic steps forming a complex biochemical network, in which multiple dietary or environmental factors (e.g., vitamin B_2, B_{12}, and alcohol) may interact with folate, therefore the genetic variations of the *MTHFR* gene alone might not be sufficient to influence colorectal tumorigenesis during the one-carbon unit metabolism. Larger studies are required to further evaluate gene-gene and gene-environment interactions in the association between *MTHFR* C677T or A1298C polymorphisms and CRC risk in Chinese populations.

4.3 Gene-environment interactions and CRC risk

Though our study was relatively smaller, we did find some evidence of interactions between the *MS* A2756G polymorphism and three environmental factors (folate intake, alcohol use, and tobacco smoking) in the CRC etiology. Our study provided the first report of an effect of the *MS* 2756 A>G polymorphism and its interactions with dietary folate intake, alcohol consumption or cigarette smoking on CRC risk in a Chinese population.

Epidemiological studies have linked heavy alcohol use to increased risk of CRC, (Giovannucci 2002; Cho, Smith-Warner et al. 2004). Because alcohol can break the folate or disturb the one-carbon unit metabolism and thus may cause abnormal DNA methylation, DNA repair, or increase the activation of precarcinogen in liver by inducing cytochrome p-450 (Giovannucci 2004; Sharp and Little 2004), drinkers carrying rare *MS* 2756G, MTHFR *677T* or 1298C alleles may have additional CRC risk caused by abnormal folate metabolism (Giovannucci 2002; Sharp and Little 2004; Matsuo, Ito et al. 2005; Yamaji, Iwasaki et al. 2009). Our results supported such an association as well as a possible interaction between these polymorphisms and alcohol use in CRC risk.

Cigarette smoking may play a role in CRC but is not a major recognized risk factor, even after a long period of exposure (Giovannucci 2001; Anderson, Attam et al. 2003), and this was also true in the present study. However, we found an interaction between *MS A2756G* genotypes and smoking; compared with non-smokers carrying the *MS* 2756AA genotype, the AG or GG carriers of heavy smokers (>20 pack-years) had a 3-fold increased CRC risk. It

was reported that *MTHFR* 677T allele caused an increased plasm homocysteine concentration in heavy smokers than in moderate or non-smokers (Brown, Kluijtmans et al. 2004), and other studies found that an interaction between smoking and *MTHFR C677T* genotypes can be determinants of adenomatous and hyperplastic polyps of colorectum (Ulvik, Evensen et al. 2001). In the present study, however, we did not find any evidence of an interaction between smoking and *MTHFR* C677T or A1298C genotypes.

Overall, as Hubner mentioned very recently (Hubner and Houlston 2009), the existing evidence is still insufficient to confirm a protective effect of folate intake, and variants of the key metabolic-enzyme genes add the complexity to the unresolved problem of how and when the folate can have an effect on CRC risk. Our results suggested that gene-environment interactions may affect CRC risk more profoundly than the individual effect of folate intake or any of other known risk factors in this study population.

There are some potential limitations in the present study. First of all, because we did not have serum levels of folate or homocysteine, there may be biases in categorizing actual folate intake levels that were solely based on questionnaire data. Second, this hospital-based case-control study may have introduced some unknown selection biases. However, we reasonably believe that our cases and controls came from the same population base served by the hospitals, because all cases were newly diagnosed and most of controls were also registered at hospitals for the first time. Third, there was inherent recall bias in case-control studies; however, our interviewers did not know case-control status of the subjects. Lastly, our relatively smaller sample size may not have sufficient study power to detect interactions among folate, alcohol, smoking and the studied genotypes on CRC risk.

5. Conclusion

The present study suggested that sufficient folate intake may reduce the risk of CRC, and alcohol use can significantly increase CRC risk in the study population. The *MS* 2756 AG+GG genotypes may be associated with an increased CRC risk; our data further suggested that the interaction between *MS* 2756 A>G polymorphism and alcohol use may result in further increased CRC risk in this Chinese populations. How and to what extent can these joint effects modify the CRC risk need additional larger epidemiological studies especially in other Chinese populations.

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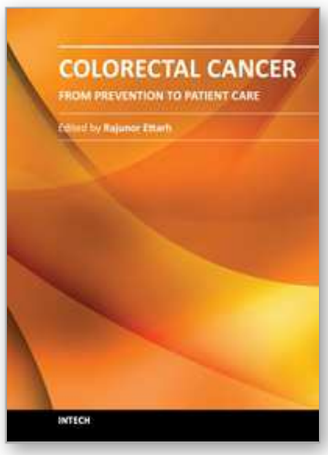

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The projections for future growth in the number of new patients with colorectal cancer in most parts of the world remain unfavorable. When we consider the substantial morbidity and mortality that accompanies the disease, the acute need for improvements and better solutions in patient care becomes evident. This volume, organized in five sections, represents a synopsis of the significant efforts from scientists, clinicians and investigators towards finding improvements in different patient care aspects including nutrition, diagnostic approaches, treatment strategies with the addition of some novel therapeutic approaches, and prevention. For scientists involved in investigations that explore fundamental cellular events in colorectal cancer, this volume provides a framework for translational integration of cell biological and clinical information. Clinicians as well as other healthcare professionals involved in patient management for colorectal cancer will find this volume useful.

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