RESEARCH ARTICLE

Folate-Related Nutrients, Genetic Polymorphisms, and Colorectal Cancer Risk: the Fukuoka Colorectal Cancer Study

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Abstract

One-carbon metabolism plays an important role in colorectal carcinogenesis. Meta-analyses have suggested protective associations of folate and vitamin B_6 intakes with colorectal cancer primarily based on studies in Caucasians, and genetic polymorphisms pertaining to the folate metabolism have been a matter of interest. Less investigated are the roles of *methionine synthase* (MTR) and *thymidylate synthetase* (TS) polymorphisms in colorectal carcinogenesis. In a study of 816 cases and 815 community controls in Japan, we investigated associations of dietary intakes of folate, methionine, vitamin B_2, vitamin B_6, and vitamin B_{12} with colorectal cancer risk. The associations with *MTR* 2756A>G, *MTRR* 66A>G, and *TSER* repeat polymorphism were examined in 685 cases and 778 controls. Methionine and vitamin B_{12} intakes were inversely associated with colorectal cancer risk, but the associations were totally confounded by dietary calcium and n-3 fatty acids. The other nutrients showed no association with the risk even without adjustment for calcium and n-3 fatty acids. The *TSER* 2R allele was dose-dependently associated with an increased risk. The *MTR* and *MTRR* polymorphisms were unrelated to colorectal cancer risk. There was no measurable gene-gene or gene-nutrient interaction, but increased risk associated with the *TSER* 2R allele seemed to be confined to individuals with high folate status. This study does not support protective associations for folate and vitamin B_6. The *TSER* 2R allele may confer an increased risk of colorectal cancer. The role of the TSER polymorphism in colorectal carcinogenesis may differ by ethnicity.

Keywords: Colorectal cancer - methionine synthase - thymidylate synthase - B-vitamins - case-control study

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Introduction

The one-carbon metabolism has drawn considerable attention in relation to colorectal carcinogenesis (Ulrich, 2005; Hubner and Houlston, 2009; Williams, 2012). A folate metabolite, 5-methyl tetrahydrofolate (THF), provides the methyl group in the reaction by *methionine synthase* (*MTR*) to convert homocysteine to methionine, the precursor of S-adenosylmethionine (SAM). The SAM is the universal methyl-group donor for methylation of a wide variety of biological substrates. Thus folate/methyl depletion results in aberrant DNA methylation, i.e., global genomic hypomethylation and specific methylation of CpG clusters in the promoters of tumor suppressor and DNA repair genes. Methylenetetrahydrofolate reductase (MTHFR) irreversibly converts 5, 10-methylene THF to 5-methyl THF. The substrate of MTHFR is required for conversion of deoxyuridylate to thymidylate by thymidylate synthase (TS). Depletion of the thymidylate pool results in uracil misincorporation into DNA, leading to single and double strand breaks (reviewed in Ulrich, 2005; Hubner and Houlston, 2009). Vitamin B_2 (riboflavin), vitamin B_6 (pyridoxine), and vitamin B_{12} (cobalamine) are involved in the key reactions in one-carbon metabolism (Ulrich, 2005). Vitamin B_2 is the precursor for a cofactor of MTHFR. Vitamin B_6 is not only a cofactor for cystathionin beta-synthase (CBS) to convert homocystein to cystathionine but also is required in the conversion of serine to glycine, recycling THF (derivative of 5-methyl THF after methyl-transfer to homocysteine) to 5, 10-methylene THF. Vitamin B_{12} is a coenzyme of *MTR*. *MTR* reductase (*MTRR*) catalyzes the reduction of

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oxidized vitamin B_{12}, releasing an activated vitamin B_{12} and *MTR*. Alcohol is also known to play an important role in folate metabolism, being a folate antagonist and an inhibitor of *MTR* (Mason and Choi, 2005).

High folate intake has been related to decreased risk of colorectal cancer (Sanjoaquin et al., 2005; Kim et al., 2010; Kennedy et al., 2011) in a series of meta-analyses and also in recent cohort (Stevens et al., 2011) and case-control (Kim et al., 2012) studies, whereas several studies have failed to find a protective association between dietary folate and colorectal cancer risk in Asia (Ishihara et al., 2007; Shrubsole et al., 2009) and Europe (Van Guelpen et al., 2006; Eussen et al., 2010). While low methionine intake, especially in combination with low folate and high alcohol intake, was related to an increased risk of colorectal cancer (Giovannucci et al., 1995; Razzak et al., 2012), methionine intake was unrelated to colorectal cancer in other studies (Harnack et al., 2002; Le Marchand et al., 2002; Ishihara et al., 2007; Murtaugh et al., 2007; Shrubsole et al., 2009). Vitamin B_2 and B_{12} have been unrelated to colorectal cancer risk in many studies of different populations (Harnack et al., 2002; Le Marchand et al., 2002; 2009; Ishihara et al., 2007; Murtaugh et al., 2007; Sharp et al., 2008; Weinstein et al., 2008; Shrubsole et al., 2009), while recent studies showed a protective association with plasma vitamin B_2 (Eussen et al., 2010) and with dietary vitamin B_2 (Zschäbitz et al., 2013). The two latter studies found no association with plasma or dietary vitamin B_{12}. On the other hand, dietary intake and plasma levels of vitamin B_6 have fairly consistently been related to decreased risk of colorectal cancer, as noted in two meta-analyses (Theodoratou et al., 2008; Larsson et al., 2010). Of the two recent large prospective studies in the United States, one reported a protective association between dietary intake of vitamin B_6 and colorectal cancer (Zschäbitz et al., 2013) while the other failed to corroborate such an association (Zhang et al., 2012).

Functional genetic polymorphisms of the key enzymes in the folate metabolism have also been a matter of interest in colorectal carcinogenesis (Sharp and Little, 2004). Two functional polymorphisms are known for MTHFR (677C>T and 1298A>C), and the variant alleles, particularly of the former, are associated with lower enzyme activities (Sharp and Little, 2004). Meta-analyses have consistently shown that MTHFR 677TT genotype is related to decreased risk of colorectal cancer in different populations (Kono and Chen, 2005; Hubner and Houlston, 2007; Huang et al., 2007; Yang et al., 2012). Results on MTHFR 1298A>C are rather variable (Kono and Chen, 2005), but MTHFR 1298CC genotype may confer a decreased risk of colorectal cancer in Caucasians (Huang et al., 2007). The *MTR* 2756A>G and *MTRR* 66A>G polymorphisms are putatively functional with respect to plasma homocysteine concentrations (Sharp and Little, 2004). Previous studies suggested a decreased risk (Ma et al., 1999; Ulvik et al., 2004) and an increased risk (Matsuo et al., 2005; de Vogel et al., 2009) of colorectal cancer associated with the *MTR* 2756GG genotype, while others found no association between the two (Le Marchand et al., 2002; Ulrich et al., 2005; Theodoratou et al., 2008; Eussen et al., 2010). Several studies have addressed the relation between *MTRR* 66A>G and colorectal cancer to find null association (Le Marchand et al., 2002; Theodoratou et al., 2008; de Vogel et al., 2009; Eussen et al., 2010). The TS gene contains polymorphic 28-bp tandem repeat sequence in the 5'-untranslated region enhancer region (*TSER*), the triple repeat (3R) showing greater gene expression than the double repeat (2R) (Sharp and Little, 2004). A decreased risk of colorectal cancer was reported among individuals homozygous for the *TSER* 2R allele (Chen et al., 2003; Ulrich et al., 2005).

We investigated associations of folate, methionine, vitamin B_2, vitamin B_6, and vitamin B_{12}, as well as of the *MTR*, *MTRR*, and TS polymorphisms, with colorectal cancer risk and effect modification of these nutrients on the association with the genetic polymorphisms including MTHFR 677C>T and 1298A>C in a community-based case-control study in Japan. We previously examined the relation of the two MTHFR polymorphisms to colorectal cancer and reported a decreased risk associated with the MTHFR 677TT genotype, particularly among those with no alcohol consumption (Yin et al., 2004).

Materials and Methods

The Fukuoka Colorectal Cancer Study is a case-control study to investigate the relation of lifestyle factors and genetic susceptibility to colorectal cancer risk in Fukuoka City and three adjacent areas, Japan. The study protocol was approved by the ethics committees of the Kyushu University Faculty of Medical Sciences and the participating hospitals. Details of the methods have been reported elsewhere (Kono et al., 2004), and methodological issues relevant to the present analysis are described below.

Subjects

Cases comprised a consecutive series of patients with histologically confirmed incident colorectal adenocarcinomas who were admitted to two university hospitals or six affiliated hospitals for surgical treatment during the period from September 2000 to December 2003. Eligible cases were aged 20-74 years at the time of diagnosis, lived in the study area, had no prior history of partial or total removal of the colorectum, familial adenomatous polyposis or inflammatory bowel disease, and were mentally competent to give informed consent and to complete the interview. Of the 1,053 eligible cases, 840 cases (80%) participated in the interview, and 685 gave informed consent for the genotyping.

Eligibility criteria for controls were the same as described for the cases except that they had no prior diagnosis of colorectal cancer. A total of 1,500 persons living in 15 small areas were selected as control candidates by a two-stage random sampling, and were invited to participate in the study by mail. Of these, 833 persons participated in the survey, and 778 gave informed consent for genotyping. The net participation rate for the interview was 60% (833/1382), with 118 persons excluded in the denominator for the following reasons: death (n=7), migration from the study area (n=22), undelivered mail (n=44), mental incompetence (n=19), history of partial or

total removal of the colorectum (n=21), and diagnosis of colorectal cancer after the survey (n=5). In the analysis on nutrients, we excluded individuals in the highest and lowest 1% of energy intake among the cases and controls combined with stratification by sex and age class (<55, 55-64, and ≥65 years) to remove those whose diet was inadequately captured by the dietary assessment. Thus 816 cases and 815 controls remained in the analysis on nutrients. Associations with the genetic polymorphisms were examined in all of the subjects who had given consent to genotyping (685 cases and 778 controls).

Interview

Research nurses interviewed cases and controls in person regarding smoking, alcohol use, physical activity, and other factors using a standardized questionnaire. Reference dates for the questionnaire were the date of onset of symptoms or screening for cases and at the time of interview for controls. Anthropometric questions inquired about height (cm), current body weight (kg), and body weight 10 years earlier. Body mass index (kg/m^2) 10 years earlier was used here, because the current body mass index was unrelated to risk (Isomura et al., 2006). Habitual alcohol consumption 5 years prior to the reference date was ascertained. The daily amount of alcohol was expressed using the conventional Japanese units; one go (180mL) of sake, one large bottle (633mL) of beer, and half a go (90mL) of shochu were each expressed as one unit; and one drink (30mL) of whisky or brandy and one glass (100mL) of wine were each converted to half a unit. The reported alcohol intake was highly reproducible (Spearman r=0.82), as tested on 29 control subjects with an interval of approximately one year.

With regard to smoking, ever-smokers were asked about duration of smoking in years and numbers of cigarettes smoked per day for each decade of life from the second to the last decade. We calculated the cumulative exposure to cigarette smoking until the beginning of the previous decade of age. Questions on physical activities elicited type of job and leisure-time activities 5 years before (Isomura et al., 2006), and the latter was expressed as a sum of metabolic equivalents (MET) multiplied by hours of weekly participation in each activity, i.e., MET-hours per week. Vitamin use was also ascertained in the interview, but the amount was not quantified.

Dietary assessment

Consumption frequencies and portion sizes of 148 food/beverage items were ascertained by a computer-assisted interview, and quantitative intake estimates were obtained for selected nutrients and food groups (Uchida et al., 2007). Individuals were asked to report their usual consumption over one year prior to the reference date. In a validation study in 28 controls, who recorded their diets over 7 days each in 4 consecutive seasons, the dietary interview was repeated with an interval of one year. Pearson correlation coefficients of energy-adjusted intakes between the first interview (and the second interview in parentheses) and diet record were as follows: folate 0.48 (0.55), methionine 0.36 (0.45), vitamin B_2 0.57 (0.49), vitamin B_6 0.61 (0.56), and vitamin B_{12} 0.13 (0.48).

Genotyping

DNA was extracted from the buffy coat using a commercial kit (Qiagen, Hilden, Germany). Both *MTR* 2756A>G (rs1805087) and *MTRR* 66A>G (rs1801394) polymorphisms were determined by the PCR-RFLP method using the primers described elsewhere (Le Marchand et al., 2002). Restriction enzyme HaeIII was applied to the 189-bp PCR product for the *MTR* 2756A>G polymorphism, and the digestion resulted in 159-bp and 30-bp fragments the 2756G allele. The *MTRR* 66A>G genotype was determined by digestion with AflIII, which cleaves the 192-bp PCR product for the 66G allele into 161-bp and 31-bp fragments. Genotypes of the *TSER* 28-bp repeat polymorphism were determined by the method described previously (Ulrich et al., 2002).

Statistical analysis

Associations of the nutrients and genetic polymorphisms with colorectal cancer risk were examined in terms of odds ratio (OR) and 95% confidence interval (CI), which were obtained from logistic regression analysis. Statistical adjustment was made for sex, 5-year age class (the lowest class of <45 years), residency area (Fukuoka City or the adjacent areas), body mass index 10 years before (<22.5, 22.5-24.9, 25.0-27.4, or ≥27.5 kg/m^2), smoking (0, 1-399, 400-799, or ≥800 cigarettes/years), alcohol intake (0, 0.1-0.9, 1.0-1.9, or ≥2.0 units/day), type of job (sedentary or non-sedentary), leisure-time physical activity (0, 1-15.9, or ≥16 MET-hours/week), and parental history of colorectal cancer. In the analysis on nutrients, further adjustment was made for dietary intakes of calcium and n-3 polyunsaturated fatty acids (PUFA), which were associated with decreased risk of colorectal cancer in the present study population (Kimura et al., 2007; Mizoue et al., 2008). Nutrient intake was adjusted to 2,000 kcal/day by the regression residual method (Willett and Stampfer, 1986). Trends in the OR were evaluated with ordinal values assigned to levels of the variable under study. The gene-gene and gene-nutrient interactions were statistically evaluated based on the likelihood ratio test, comparing the model including a term or terms for interaction with the model without. Statistical significance was declared if two-sided P was less than 0.05. Statistical analyses were carried out using SAS version 9.2 (SAS Institute, Cary, NC).

Results

Table 1 shows selected characteristics of colorectal cancer cases and controls. Cases were slightly older than controls, had higher body mass index, more sedentary in their job, and physically more active in leisure time. Heavy alcohol use and parental colorectal cancer were more frequent in cases than in controls. No significant difference was observed between cases and controls with respect to smoking and total energy intake. Average daily intakes of methionine and vitamin B_{12} were lower in cases than in controls. There was no measurable difference in the intake of folate, vitamin B_2, or vitamin B_6 between cases and controls.

In cases and controls combined, main sources of

folate intake were vegetables (36%) and green tea (16%) followed by cereals (7%), fruits (7%), and liver (7%). Methionine were derived largely from fish and shellfish (29%), meats (19%), and cereals (22%). Vitamin B_2 intake was derived from a variety of animal and plant foods including cereals (11%), vegetables (8%), legumes (7%), green tea (13%), fish and shellfish (12%), meats and liver (12%), and milk and dairy products (13%). Dietary sources of vitamin B_6 were also diverse, main foods being vegetables (21%), fruits (10%), fish and shellfish (20%), and meats (12%). The majority of vitamin B_{12} intake was due to consumptions of fish and shellfish (71%) and meats (16%). Calcium intake was positively correlated with each of the nutrients under study, with Spearman r ranged 0.47-0.66 in the control group, and n-3 PUFA intake was most strongly correlated with methionine (Spearman r 0.63) and vitamin B_{12} (Spearman r 0.56).

Higher intakes of methionine and vitamin B_{12} were each associated with a decreased risk of colorectal cancer with a statistically significant trend when the dietary factors were not taken into account (Table 2). After adjustment for intakes of calcium and n3-PUFA, the inverse associations with methionine and vitamin B_{12} disappeared. Intakes of folate, vitamin B_2, and vitamin B_6 were unrelated to colorectal cancer risk regardless of adjustment for calcium and n3-PUFA intakes.

Regarding the *TSER* polymorphism, 25 cases and 31 controls had alleles of greater than 3 repeats, genotypes of such alleles were 2R/4R (one control), 2R/5R (6 cases and 6 controls), 3R/4R (4 controls), 3R/5R (18 cases and 20 controls), and 5R/5R (one case). Frequencies of the *MTR* 2756G, *MTRR* 66G, and *TSER* 2R among the cases were 0.184, 0.298, and 0.173, respectively. The corresponding frequencies in the controls were 0.178, 0.316, and 0.138, respectively. The distribution of genotypes of each polymorphism was in agreement with the Hardy-Weinberg equilibrium in cases and controls each.

Table 3 summarizes crude and adjusted OR of colorectal cancer in relation to the *MTR*, *MTRR*, and *TSER* polymorphisms. The OR increased with an increasing number of the *TSER* 2R allele, showing a statistically significant trend, regardless of adjustment for the covariates. The association with this polymorphism was essentially the same when *TSER* 3R, 4R, and 5R alleles were collapsed into one group (3+R allele); the adjusted OR for the 3+R/2R and 2R/2R genotypes as compared with the 3+R/3+R genotype were 1.28 (95%CI 1.01-1.64) and 1.81 (95%CI 0.92-3.56), respectively (p_{trend}=0.01), and the adjusted OR for the 3+R/2R and 2R/2R genotypes combined were 1.32 (95%CI 1.05-1.67). Neither the *MTR* nor *MTRR* polymorphism showed a measurable association with colorectal cancer. Adjusted OR for individuals with the *TSER* 2R allele were 1.27 (95% CI 0.96-1.70) for colon cancer and 1.37 (95% CI 1.01-1.86)

Table 1. Selected Characteristics of Cases of Colorectal Cancer and Controls

Variable	Cases (n=816)	Controls (n=815)	$p_{difference}$*
Men (%)	59.8	61.8	0.40
Age (year), mean	60.5	58.9	0.0006
Body mass index (kg/m^2), mean†	23.3	22.9	0.02
Ever smoking (%)‡	54.4	56.1	0.36
Alcohol use (%)‡	58.7	58.3	0.84
High alcohol use, ≥1 units/day (%)‡	38.8	34.2	0.03
Non-sedentary job (%)‡	26.3	29.3	0.17
Leisure-time activity, ≥16 MET-hr/week (%)‡	32.1	36.2	0.08
Parental colorectal cancer (%)‡	7.9	5.4	0.04
Energy intake (kcal/day), geometric mean†	2154	2146	0.76
Micronutrient intake, geometric mean†§			
Folate (μg/day)	355	355	0.91
Methionine (mg/day)	1437	1477	0.002
Vitamin B_2 (mg/day)	1.43	1.44	0.74
Vitamin B_6 (mg/day)	1.28	1.29	0.70
Vitamin B_{12} (μg/day)	7.74	8.02	0.09

*Unpaired t-test for mean and chi-square test for proportions, unless otherwise specified; †Adjusted for sex and 5-year age class by analysis of covariance; ‡Adjusted for sex and age class (<55, 55-64, and >65 years) by the direct method with numbers of cases and controls combined as standard population. $p_{difference}$ was based on the Mantel-Haenszel method; §Energy-adjusted intake per 2000 kcal/day based on the regression residual method

Table 2. Colorectal Cancer Risk According to Energy-adjusted Intakes of Folate, Methionine, and Vitamin B

Micronutrient		Quintiles of dietary intake					p_{trend}
		Q1 (low)	Q2	Q3	Q4	Q5 (high)	
Folate (μg/day)	Median intake	234	300	355	417	522	
	n*	155/163	159/163	151/163	182/163	169/163	
	OR (95% CI)†	1.00	0.95 (0.69–1.31)	0.90 (0.65–1.24)	1.03 (0.75–1.43)	0.93 (0.66–1.32)	0.90
	OR (95% CI)‡	1.00	0.98 (0.71–1.37)	0.96 (0.68–1.35)	1.18 (0.83–1.68)	1.14 (0.78–1.68)	0.31
Methionine (mg/day)	Median intake	1195	1351	1483	1608	1822	
	n*	192/163	169/163	146/163	143/163	166/163	
	OR (95% CI)†	1.00	0.90 (0.66–1.23)	0.74 (0.54–1.02)	0.69 (0.50–0.95)	0.77 (0.56–1.07)	0.04
	OR (95% CI)‡	1.00	0.91 (0.66–1.26)	0.79 (0.56–1.12)	0.79 (0.54–1.15)	1.04 (0.69–1.57)	0.82
Vitamin B_2 (mg/day)	Median intake	1.09	1.29	1.45	1.61	1.91	
	n*	177/163	154/163	141/163	169/163	175/163	
	OR (95% CI)†	1.00	0.86 (0.63–1.18)	0.83 (0.60–1.15)	0.88 (0.65–1.21)	0.93 (0.67–1.28)	0.72
	OR (95% CI)‡	1.00	0.87 (0.63–1.22)	0.90 (0.63–1.28)	0.99 (0.69–1.43)	1.18 (0.80–1.74)	0.29
Vitamin B_6 (mg/day)	Median intake	0.97	1.16	1.29	1.44	1.71	
	n*	149/163	173/163	150/163	191/163	153/163	
	OR (95% CI)†	1.00	1.09 (0.79–1.50)	0.87 (0.63–1.22)	1.16 (0.83–1.61)	0.88 (0.63–1.25)	0.66
	OR (95% CI)‡	1.00	1.15 (0.83–1.60)	0.96 (0.68–1.37)	1.38 (0.96–1.98)	1.15 (0.78–1.71)	0.30
Vitamin B_{12} (μg/day)	Median intake	4.52	6.50	8.17	10.04	13.49	
	n*	166/163	180/163	171/163	150/163	149/163	
	OR (95% CI)†	1.00	1.08 (0.79–1.48)	0.97 (0.71–1.34)	0.81 (0.59–1.12)	0.76 (0.55–1.06)	0.03
	OR (95% CI)‡	1.00	1.14 (0.82–1.57)	1.07 (0.76–1.50)	0.92 (0.64–1.32)	0.96 (0.65–1.43)	0.50

*Number of cases/controls; †Adjusted for sex, age (5-year class), resident area, cigarette smoking, alcohol consumption, body mass index, type of job, leisure-time physical activity, and parental colorectal cancer; ‡Further adjusted for dietary intakes of calcium and n-3 polyunsaturated fatty acids (quintiles)

Table 3. *MTR*, *MTRR* and *TSER* Polymorphisms and Colorectal Cancer Risk

Genotype		Cases No. (%)	Controls No. (%)	Crude OR (95% CI)		Adjusted OR (95% CI)*	
MTR 2756A>G†	AA	449 (65.6)	525 (67.6)	1.00 (referent)		1.00 (referent)	
	AG	218 (31.9)	228 (29.3)	1.12 (0.89-1.40)		1.12 (0.89-1.41)	
	GG	17 (2.5)	24 (3.1)	0.83 (0.44-1.56)	p_{trend}=0.64	0.81 (0.42-1.55)	p_{trend}=0.66
	AG+GG	235 (34.4)	252 (32.4)	1.09 (0.88-1.36)		1.09 (0.87-1.37)	
MTRR 66A>G	AA	342 (49.9)	361 (46.4)	1.00 (referent)		1.00 (referent)	
	AG	278 (40.6)	343 (44.1)	0.86 (0.69-1.06)		0.83 (0.67-1.04)	
	GG	65 (9.5)	74 (9.5)	0.93 (0.64-1.34)	p_{trend}=0.30	0.90 (0.62-1.31)	p_{trend}=0.21
	AG+GG	343 (50.1)	417 (53.6)	0.87 (0.71-1.07)		0.85 (0.69-1.04)	
TSER 26-bp repeat‡	3R/3R	451 (68.3)	555 (74.3)	1.00 (referent)		1.00 (referent)	
	3R/2R	187 (28.3)	176 (23.6)	1.31 (1.03-1.66)		1.29 (1.01-1.65)	
	2R/2R	22 (3.3)	16 (2.1)	1.69 (0.88-3.26)	p_{trend}=0.01	1.80 (0.92-3.54)	p_{trend}=0.01
	3R/2R+2R/2R	209 (31.7)	192 (25.7)	1.34 (1.06-1.69)		1.33 (1.05-1.68)	

*Adjusted for sex, age, resident area, cigarette smoking, alcohol consumption, body mass index, type of job, leisure-time physical activity, and parental colorectal cancer; †Genotype was undetermined for one case and one control; ‡Subjects with 4- or 5-repeat allele were excluded (25 cases and 31 controls)

Table 4. Joint Effects of *MTHFR* TT Genotype and *MTR*, *MTRR* and *TSER* Genotypes on Colorectal Cancer Risk

Genotype	*MTHFR* 677C>T				$p_{interaction}$
	CC+CT		TT		
	n*	OR (95% CI)†	n*	OR (95% CI)†	
MTR 2756A>G					
AA	401/438	1.00 (referent)	48/87	0.62 (0.42-0.91)	0.30
AG+GG	198/206	1.05 (0.82-1.34)	37/46	0.90 (0.56-1.42)	
MTRR 66A>G					
AA	295/285	1.00 (referent)	47/76	0.63 (0.42-0.95)	0.54
AG+GG	305/360	0.81 (0.64-1.02)	38/57	0.62 (0.39-0.97)	
TSER 26-bp repeat					
3R/3R	397/464	1.00 (referent)	54/91	0.71 (0.49-1.03)	0.99
3R/2R+2R/2R	183/158	1.33 (1.03-1.72)	26/34	0.94 (0.55-1.62)	

*Number of cases/controls; †Adjusted for sex, age, resident area, cigarette smoking, alcohol consumption, body mass index, type of job, leisure-time physical activity, and parental colorectal cancer

for rectal cancer. There was no distinct difference in the association between colon and rectal cancers with respect to the other polymorphisms.

In the analysis on the gene-gene interaction, heterozygotes were combined with the variant homozygotes, because the latter were generally limited in number. For MTHFR 677C>T, however, the CC and CT genotypes were combined, because decreased risk are confined to the TT genotype (Kono and Chen, 2005; Huang et al., 2007; Hubner et al., 2007). There was no evident interaction between any two of the polymorphisms. For instance, a decreased risk associated with the MTHFR 677TT genotype was consistently observed across genotype strata of the other polymorphisms (Table 4). Only a suggestive interaction was noted for *MTRR* 66A>C and MTHFR 1298A>C (p=0.07); an increased risk was observed among those with the MTHFR 1298C allele and *MTRR* 66AA genotype compared with those having the MTHFR 1298AA and *MTRR* 66AA genotype (adjusted OR 1.42, 95%CI 1.02-1.95).

The association between each genetic polymorphism and colorectal cancer risk was examined with nutrient intake stratified into tertile categories. Neither *MTR* 2756A>G nor *MTRR* 66A>G polymorphism showed an increase or decrease in the OR for any specific strata of the nutrient and alcohol intake (data not shown). On the other hand, although the interaction was far from the statistical significance, an increased risk associated with the *TSER* 2R allele was observed in individuals with

Table 5. Adjusted Odds Ratios (95% confidence intervals) of Colorectal Cancer for *TSER* Genotypes, with Stratification by Dietary Intake of Folate-related Nutrient and Alcohol

Nutrient/genotype	Dietary intake*			$p_{interaction}$
	Low	Intermediate	High	
Folate				
3R/3R	1.00 (referent)	1.12 (0.80-1.57)	1.09 (0.75-1.60)	0.37
3R/2R+2R/2R	1.07 (0.68-1.68)	1.43 (0.93-2.20)	1.80 (1.15-2.82)	
Methionine				
3R/3R	1.00 (referent)	0.94 (0.67-1.32)	0.89 (0.59-1.35)	0.28
3R/2R+2R/2R	1.18 (0.77-1.81)	1.08 (0.70-1.67)	1.58 (0.99-2.54)	
Vitamin B_2				
3R/3R	1.00 (referent)	0.92 (0.65-1.30)	1.04 (0.70-1.54)	0.36
3R/2R+2R/2R	1.05 (0.70-1.59)	1.38 (0.86-2.20)	1.61 (1.01-2.58)	
Vitamin B_6				
3R/3R	1.00 (referent)	1.00 (0.71-1.41)	1.16 (0.79-1.69)	0.62
3R/2R+2R/2R	1.30 (0.85-1.99)	1.18 (0.77-1.82)	1.83 (1.15-2.91)	
Vitamin B_{12}				
3R/3R	1.00 (referent)	1.10 (0.79-1.53)	1.02 (0.70-1.50)	0.53
3R/2R+2R/2R	1.45 (0.94-2.24)	1.64 (1.08-2.48)	1.11 (0.69-1.78)	
Alcohol†				
3R/3R	1.00 (referent)	1.13 (0.79-1.61)	1.46 (1.03-2.06)	0.18
3R/2R+2R/2R	1.79 (1.22-2.63)	1.28 (0.78-2.10)	1.62 (1.06-2.49)	

*Intake was categorized at tertiles in the control group, except for alcohol intake (0, <1, and ≥1 units/day); †Adjusted for sex, age, area, cigarette smoking, alcohol consumption (for nutrients), body mass index, type of job, physical activity, parental colorectal cancer, and dietary intakes of calcium and n3-polyunsatruated fatty acids

high intake of folate, methionine, vitamin B_2, or vitamin B_6 and in those with no consumption of alcohol (Table 5). Decreased risk associated with the MTHFR 677TT genotype seemed to be more evident when vitamin B_2 intake was lower ($p_{interaction}$=0.40). As compared with those having the MTHFR 677CC or CT genotype who were in the lowest tertile of vitamin B_2, adjusted OR for the 677TT genotype in the low, intermediate, and high tertiles were 0.54 (95%CI 0.32-0.92), 0.66 (0.38-1.15), and 1.03 (0.59-1.81), respectively. No other nutrients modified the association with the MTHFR 677TT genotype, while a decrease in the OR with the MTHFR 677TT genotype was more evident among non-alcohol drinkers.

Discussion

Dietary intakes of methionine and vitamin B_{12} were associated with a decreased risk of colorectal cancer risk, but the associations were confounded by dietary intakes of calcium and n-PUFA. Dietary intakes of folate, vitamin

B_2, and vitamin B_6 showed no association with colorectal cancer risk even when intakes of calcium and n-PUFA were not taken into consideration. The *TSER* 2R allele was dose-dependently associated with an increased risk of colorectal cancer, the increase being more evident when intake of folate, methionine, vitamin B_2, or vitamin B_6 was high or when alcohol was not consumed.

The present study did not show a protective association with either folate or vitamin B_6 in a Japanese population. Use of folate and vitamin B_6 supplements was not considered in the present study. However, non-dietary intakes of these vitamins were probably small in the study population. Foods are not enriched with folic acid in Japan. No more than 64 subjects (31 cases and 33 controls) reported that they had used specific vitamin B complex or B-containing multivitamins daily at least for 5 years, and none of these specified multivitamins contained folic acid. Thus folate or vitamin B_6 intake may have been lower in the present population than in North America, where folate fortification is mandatory and supplements are commonly used (Kim, 2008). While an earlier meta-analysis suggested that dietary folate intake, rather than total folate intake, was more strongly related to a decreased risk of colorectal cancer (Sanjoaquin et al., 2005), a recent pooled analysis of prospective studies in North America and Europe indicated that the decrease in colorectal cancer risk was greater for total folate intake than for dietary folate intake (Kim et al., 2010). As for vitamin B_6, supplement use accounted for the majority of total intake and resulted in a wider range of the distribution of the intake in some study populations in North America (Larsson et al., 2010). Nonetheless, dietary intake of vitamin B_6 was shown to be related to a decreased risk of colorectal cancer in different populations worldwide, although the findings are not necessarily consistent (Theodoratou et al., 2008; Larsson et al., 2010; Zhang et al., 2012; Zschäbitz et al., 2013). The findings on methionine, vitamin B_2 and vitamin B_{12} are in agreement with the observation in the majority of the previous studies (Harnack et al., 2002; Le Marchand et al., 2002; 2009; Ishihara et al., 2007; Murtaugh et al., 2007; Sharp et al., 2008; Weinstein et al., 2008; Shrubsole et al., 2009).

The present findings on the *TSER* polymorphism are contrary to the results from the studies in the United States (Chen et al., 2003; Ulrich et al., 2005), in which individuals with the *TSER* 2R/2R genotype showed a decreased risk of colorectal cancer. It is known that patients of colorectal cancer with the *TSER* 3R/3R genotype respond less to 5-FU-based chemotherapy (Winder and Lenz, 2010), but it remains uncertain how the TS activity is involved in colorectal carcinogenesis. It is possible that the role for the *TSER* polymorphism in folate metabolism may differ in different ethnicities. The *TSER* 2R allele is very common in Caucasians (Chen et al., 2003; Ulrich et al., 2005), but rare in Asians (Trinh et al., 2002). The *TSER* 2R allele was associated with lower concentrations of plasma folate in Caucasians (Chen et al., 2003), but with higher folate concentrations in Chinese (Trinh et al., 2002). Decreased risk of colorectal cancer associated with the MTHFR 677TT genotype is putatively ascribed to proper DNA synthesis and repair due to a greater supply of methyleneTHF (Ulrich, 2005). Thus an increased risk associated with the *TSER* 2R allele is mechanistically compatible with the folate-related pathway in colorectal carcinogenesis. Interestingly, an increased risk associated with the *TSER* 2R allele was pronounced in the high folate status. The findings are seemingly paradoxical, but the effect of lowered TS expression could be more discernible when the supply of methleneTHF is sufficient.

The present study showed that decreased risk with the MTHFR 677TT genotype was more evident when vitamin B_2 intake was lower. Again, the finding is contradictory to the observation in the United States; a decreased risk with the MTHFR 677TT genotype was observed in the second and third tertiles, not in the lowest tertile, of dietary vitamin B_2 in the Multiethnic Cohort study (Le Marchand et al., 2005). Low levels of vitamin B_2 further reduce the enzyme activity, and thereby accentuating the pool of methlene THF. In earlier prospective studies in the United States (Chen et al., 1996; Ma et al., 1997), a decreased risk of colorectal cancer with the MTHFR 677TT genotype was observed specifically among individuals with high folate intake. While the initial observation was replicated in the Multiethnic Cohort study (Le Marchand et al., 2005; 2009), such an effect modification has not been confirmed in other studies in the United States and other countries (Kono and Chen, 2005). A decreased risk associated with MTHFR 677TT genotype or the 677CT/TT genotype was more pronounced in those with low folate intake in Scotland (Sharp et al., 2008) and Japan (Matsuo et al., 2005). The MTHFR 677TT genotype was unrelated to colorectal cancer risk regardless of plasma folate levels in the EPIC study (Eussen et al., 2010). On the other hand, a decreased risk associated with the MTFR 677TT genotype has been more consistently observed among those with null or modest alcohol consumption (Kono and Chen, 2005; Le Marchand et al., 2005; Sharp et al., 2008).

The lack of an association between *MTR* 2756A>G and colorectal cancer in the present study is in agreement with the findings in some of the previous studies (Harnack et al., 2002; Ulrich et al., 2005; Theodoratou et al., 2008; Eussen et al., 2010), but not in others (Ma et al., 1999; Ulvik et al., 2004; Matsuo et al., 2005; de Vogel et al., 2009). A recent meta-analysis concluded that *MTR* 2756A>G was unrelated to colorectal cancer risk (Ding et al., 2013). The *MTR* 2756GG genotype was associated with a decreased risk of colorectal cancer in individuals with high plasma folate or low alcohol consumption (Ma et al., 1999) and in those with low concentrations of plasma homocysteine (Ulvik et al., 2004). Another study showed an increased risk for the *MTR* 2756GG genotype among alcohol drinkers (Matsuo et al., 2005). We did not separate the *MTR* 2756GG genotype in the analysis on the gene-nutrient interaction because individuals with the *MTR* 2756GG genotype were very few. In a repeated analysis separating the *MTR* 2756GG genotype, however, there was no effect modification of alcohol on the association with the *MTR* 2756GG genotype. Adjusted OR for alcohol consumptions of 0, <1, and 1 units/day among those with the *MTR* 2756GG genotype were 1.20 (95%CI 0.49-2.93), 0.73 (0.06-8.94), and 0.72 (0.23-2.28), respectively, as compared with non-alcohol drinkers with *MTR* 2756AA

genotype ($p_{interaction}$=0.73). The null association with the *MTRR* 66A>G is consistent with the previous observation in a limited number of studies in Caucasians (Le Marchand et al., 2002; Theodoratou et al., 2008; de Vogel et al., 2009; Eussen et al., 2010).

The use of community controls were among strengths of the present study. The frequencies of the *MTR* 2756G, *MTRR* 66G, and *TSER* 2R alleles in the control group were very similar to those reported elsewhere in Japan (Matsuo et al., 2005). A limitation of the present study was that the participation rate in terms of the genotyping was not optimal. However, the participation was relatively similar in cases (65%) and controls (56%), and it is unlikely that the participation was associated with genotype and thus that selection bias occurred with respect to the polymorphisms under study. Another limitation was the retrospective assessment of intake of folate-related nutrients and alcohol consumption. Recent dietary intake may not have captured the intakes directly relevant to the development of colorectal cancer. Finally, although the total number of subjects was fairly large, the sample size was much smaller in the analyses on the gene-gene and gene-nutrient interactions.

In summary, a case-control study in Japan showed no association of dietary intake of folate and related nutrients with colorectal cancer and showed a modest increase in the risk associated with the *TSER* 2R allele.

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