Risk of colorectal cancer associated with the C677T polymorphism in 5,10-methylenetetrahydrofolate reductase in Portuguese patients depends on the intake of methyl-donor nutrients[1–3]

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ABSTRACT

Background: Polymorphisms located in genes involved in the metabolism of folate and some methyl-related nutrients are implicated in colorectal cancer (CRC).

Objective: We evaluated the association of 3 genetic polymorphisms [C677T *MTHFR* (methylene tetrahydrofolate reductase), A2756G *MTR* (methionine synthase), and C1420T *SHMT* (serine hydroxymethyltransferase)] with the intake of methyl-donor nutrients in CRC risk.

Design: Patients with CRC ($n = 196$) and healthy controls ($n = 200$) matched for age and sex were evaluated for intake of methyl-donor nutrients and the 3 polymorphisms.

Results: Except for folate intake, which was significantly lower in patients ($P = 0.02$), no differences were observed in the dietary intake of other methyl-donor nutrients between groups. High intake of folate (>406.7 μg/d) was associated with a significantly lower risk of CRC (odds ratio: 0.67; 95% CI: 0.45, 0.99). The A2756G *MTR* polymorphism was not associated with the risk of developing CRC. In contrast, homozygosity for the C677T *MTHFR* variant (TT) presented a 3.0-fold increased risk of CRC (95% CI: 1.3, 6.7). Similarly, homozygosity for the C1420T *SHMT* polymorphism also had a 2.6-fold increased risk (95% CI: 1.1, 5.9) of developing CRC. When interactions between variables were studied, low intake of all methyl-donor nutrients was associated with an increased risk of CRC in homozygous participants for the C677T *MTHFR* polymorphism, but a statistically significant interaction was only observed for folate (odds ratio: 14.0; 95% CI: 1.8, 108.5). No significant associations were seen for *MTR* or *SHMT* polymorphisms.

Conclusion: These results show an association between the C677T *MTHFR* variant and different folate intakes on risk of CRC. *Am J Clin Nutr* 2008;88:1413–8.

INTRODUCTION

Colorectal cancer (CRC) is a complex disease that involves multiple genetic and nutritional factors (1). Among the latter, folate was shown to play a preventive role in colorectal carcinogenesis probably because of its involvement in the processes of DNA methylation and synthesis (2). Other nutrients such as methionine, vitamin B-6, and vitamin B-12, which interact metabolically with folate in this process, may also influence the risk of CRC (3). In some of those studies the observed inverse association between folate status and CRC risk was further modified by genetic polymorphisms of the enzymes involved in folate metabolism, most notably methylene tetrahydrofolate reductase (MTHFR). A common C677T substitution in the *MTHFR* gene, converting alanine to valine, results in a thermolabile enzyme with decreased activity (4). Numerous studies have shown that this variant (TT) is associated to a decreased risk of CRC, but only when folate status is normal or high. As stated by Crott and Mason (4), *MTHFR* polymorphism is possibly the only known gene polymorphism that switches from being a risk factor to a protective one depending on nutrient status. In addition, because folate metabolism involves the interconversion of different coenzymatic forms of the vitamin through multiple cycles, as well as feedback mechanisms between cycles, it is also important to evaluate the joint influence that other polymorphisms in genes involved in these cycles might exert. Methionine synthase requires vitamin B-12, as methylcobalamin, as a cofactor. A variant in this gene was described, A2756G, which results in the substitution of aspartate by glycine. Studies on colorectal neoplasia are inconsistent, with some studies showing that GG genotype is associated to a decreased risk of CRC (5, 6), yet a possible increased risk of colorectal adenoma (7). No associations with diet were sought in those previous studies. Serine hydroxymethyltransferase (SHMT) is a pyridoxal phosphate (B6)–dependent enzyme. A polymorphism was identified, C1420T, which results in the substitution of the amino acid leucine by phenylalanine. The functional significance of this polymorphism is still unknown. A single study has examined the influence that this polymorphism might have in the risk of CRC and found no association. Again, no associations with dietary intake were examined (8). Therefore, it was the aim of this case-control study to evaluate the role of these polymorphisms in

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defining CRC risk either alone or in association with specific nutrient intakes and other genotypes.

SUBJECTS AND METHODS

Study population

Study participants were described previously (9). Briefly this was a case-control study, performed at the Instituto Português de Oncologia de Lisboa Francisco Gentil EPE. The study was approved by the Scientific and Ethics committees of the Instituto Português de Oncologia de Lisboa Francisco Gentil EPE, and both patients and controls gave their informed written consent to participate in the study.

The patients' group is composed of 196 subjects [104 men, 92 women; age ($\bar{x} \pm$ SD): 64.2 ± 11.3 y) with a histologic diagnosis of CRC, whereas the control group included 200 healthy blood donors volunteers recruited from the same institute, with a similar sex and age distribution (106 men, 94 women; mean age: 62.2 ± 12.1 y) and with no previous history of cancer at any site. Colonoscopy was not performed in controls to exclude CRC, but all subjects were symptom free, and no anemia was present. One hundred sixty-nine (86.5%) of 196 patients had a recent diagnosis of CRC, whereas the remaining 27 (13.5%) were being treated for a disease relapse. For previous therapies, 119 (60.7%) of 196 patients had not received any form of treatment, 28 (14.3%) had already undergone surgery, 13 (6.6%) had received pelvic radiotherapy, 11 (5.6%) had received ≥1 cycles of chemotherapy, and the remaining 25 (12.8%) had undergone combined forms of treatment. Tumor-node-metastasis staging was as follows: stage I, 24 (13.5%) of 178; stage II, 64 (35.9%) of 178; stage III, 53 (29.8%) of 178; and stage IV, 37 (20.8%) of 178.

Nutritional intake evaluation

For quantifying folate, vitamin B-6, vitamin B-12, glycine, methionine, serine, and alcohol intake, we used a food-frequency questionnaire (10), validated for the Portuguese population. Participants were asked to recall their habits in the year before CRC diagnosis in patients or in the year before interview in controls. Colored photographs of most food items, showing 3 different portions sizes, as well as measuring cups and spoons, were used to facilitate quantification of intake. Type and quantity of food intake was then analyzed in a modified database FOOD PROCESSOR SOFTWARE, version 7 (ESHA Research Inc, Salem, OR), including some Portuguese food items, which allows the quantification of different macronutrients and micronutrients. Nutrient values were calculated from foods and supplements (11 of 196 patients and none of the controls were taking supplements). Physical activity in the year before cancer diagnosis in patients or in the year before interview in controls was assessed with the use of Jackson's questionnaire (11), which categorized subjects in 3 different levels: low (0), intermediate (1), and high (2). Smoking habits were recorded as the number of cigarettes per day.

Genotype data

Whole blood samples were collected and stored in 3-mm Cards Kit papers. DNA extraction was done with the use of Generation Capture Card Kit, DNA Purification, DNA Elution (Gentra Systems Inc, Minneapolis, MN). The GAC/GTC polymorphism at codon 677 of the *MTHFR* gene and the A2756G polymorphism from *MTR* (5,10-methylenetetrahydrofolate reductase) were genotyped with the use of an "Assays-by-Design" TaqMan allelic discrimination assay (Applied Biosystems, Foster City, CA). The *SHMT* polymorphism C1420T was genotyped with the use of TaqMan probes designed with BEACON DESIGNER 5.0 software (Premier Biosoft, Palo Alto, CA). Probes for the C allele (CAGAGGGAAGAGAGAGGCGAAGC) and T allele (CAGAGGGAAGAAAGAGGCGAAGC) and for the forward primer (GAAAGAGTTCAAGGAGAGACT) and the reverse primer (CTCCTTTAGAAGTCAGGCAG) were used. Polymerase chain reaction (PCR) was performed with mixes consisting of 4 μL of genomic DNA, 10 μL of Taqman Universal PCR Master Mix (Applied Biosystems), 1 μL of 20× assay mix, and ddH_2O ≤20 μL of final volume. The following amplification protocol was used: denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 92 °C for 15 s, and annealing and extension at 60 °C for 1 min. All the reactions were performed in the iCycler iQ Multicolor Real Time PCR Detection System (Bio-Rad, Hercules, CA). After PCR, the genotype of each sample was attributed automatically by measuring the allelic-specific fluorescence on the equipment software.

Subjects were classified as homozygous for the variant if they carried 2 mutated alleles, heterozygous if they carried only 1 mutated allele, and finally homozygous for the wild type when they had no mutant alleles. When analyzing the interaction between genetic and nutritional variables, the median value for each nutrient was considered. We did not use the dietary reference intake because, for most nutrients, the percentage of subjects who ingested the dietary reference intake was higher than 80%, for both patients and controls.

Statistical methods

Statistical analysis was performed with the use of SPSS version 15.0 for WINDOWS (SPSS Inc, Chicago, IL). Data were expressed as mean ± SD, as number of subjects and (percentage), or as odds ratio (OR) and 95% CI. Bivariate analyses were conducted with the use of the Student's *t* test or Mann-Whitney test for continuous and the chi-square test for categorical variables. Multiple logistic regression was used to assess the variables related to CRC risk. Significance of interactions was assessed with the likelihood ratio test comparing the model with interaction with one containing only the main effects for 2 variables. Statistical significance was established at $P < 0.05$.

RESULTS

Subjects characteristics and lifestyle habits

Both groups were similar for age and sex distribution. Some patients (23%) had a positive familial history of CRC in first- or second-degree relatives, contrasting with only 8% of the control population ($P < 0.001$; OR: 3.4; 95% CI: 1.85, 6.29). No differences were found in smoking habits between the groups ($P > 0.05$), but compared with controls, patients had a significantly lower body mass index (in kg/m^2: 27.0 ± 3.9 compared with 25.6 ± 4.2; $P = 0.01$) and were significantly less active ($P = 0.001$). However, exercise per se did not influence the risk of developing CRC (OR: 0.78; 95% CI: 0.53, 1.1).

Nutritional intake evaluation

The daily nutritional intake of folate, vitamins B-12 and B-6, glycine, methionine, serine, and alcohol is shown in **Table 1**. No significant differences were observed between cases and controls except for folate intake that was significantly lower in patients ($P = 0.02$). Analyzing the risk associated with this nutrient intake, we observed a risk (OR) of 0.67 (95% CI: 0.45, 0.99) for participants with a high intake of folate (>406.7 μg/d). Alcohol consumption was higher in patients, but the difference did not reach statistical significance, because 35.2% of the patients and 38.5% of the controls did not drink any type of alcoholic beverage. However, if we consider only alcohol drinkers in both groups, we observed that daily intake was significantly higher in patients than in controls: 38.8 g/d compared with 31.0 g/d, respectively (OR: 1.97; 95% CI: 1.19, 3.26). When analyzing the concomitant influence of ≥2 nutrients in modulating the risk of CRC, no interaction between variables was found, except for a high alcohol intake combined with low folate ingestion, which was associated, to a statistically marginal degree, with a higher risk of developing CRC (OR: 1.47; 95% CI: 0.94, 2.30; $P = 0.08$).

Polymorphism genotypes

The genotype frequencies for polymorphisms are reported in **Table 2**. Genotype distributions among controls were in agreement with the Hardy Weinberg equilibrium with the exception of the C677T *MTHFR* polymorphism. The variant allele frequencies for C677T *MTHFR*, A2756G *MTR*, and C1420T *SHMT* genotypes were 31.2%, 16.7%, and 26.5%, respectively, in controls, and 32.7%, 15.1%, and 28.6%, respectively, in cases. A2756G *MTR* per se was not associated with increased risk of CRC (Table 2). In contrast, homozygous participants for the C677T *MTHFR* variant (TT) showed a 3.0-fold increased risk of CRC (OR: 3.01; 95% CI: 1.3, 6.7). Similarly, homozygosity for the C1420T *SHMT* polymorphism had a 2.6-fold increased risk (95% CI: 1.1, 5.9) of developing CRC. None of the variant alleles of the 3 polymorphisms was associated with the risk of CRC.

Considering only the patient group, we did not observe any correlation between genotype and sex, tumor location (colon or rectum), stage of disease, or familial history of CRC. We also evaluated the interaction between the 3 polymorphisms in modulating the risk of CRC. For each sex no significant interaction was observed between the different genotypes (data not shown).

Diet and polymorphisms interaction

The interaction between the intake of several methyl-donor nutrients and alcohol and the C677T *MTHFR* polymorphism is shown in **Table 3**. Interestingly, we observed that a low intake of all methyl-donor nutrients was associated with increased risk of developing CRC in TT carriers, but a significant interaction was only observed for folate (OR: 14.0; 95% CI: 1.8, 108.5). For alcohol intake no modulating effect was observed. For the A2756G *MTR* and C1420T *SHMT* polymorphisms, no significant interactions were observed with any of the nutrients studied.

DISCUSSION

The present case-control study focused on the risk of CRC and the interaction between intake of folate and other methyl-related nutrients and genetic polymorphisms in folate-metabolizing enzymes in a Portuguese population. A number of epidemiologic, clinical, and animal studies performed during the past 2 decades strongly support the concept that low folate status predisposes to colorectal carcinogenesis (12). Biological mechanisms linking folate depletion to colorectal carcinogenesis include disruption of nucleotide synthesis, DNA methylation, or both because folate is the primary intermediary for most methylation reactions in cellular metabolism (2, 12–15).

Although most studies focus on folate alone, there are other nutrients, such as vitamin B-6 or vitamin B-12, which are important coenzymes required for the activity of different enzymes involved in these methylation cycles such as MTR or SHMT, or certain essential and nonessential amino acids that also play important functions in the DNA methylation cycle. For these reasons, in the present study, we evaluated the influence of these methyl-related nutrients either alone or in interaction with the folate-metabolizing enzymes MTHFR, MTR, and SHMT in the risk of CRC.

Our data show that, except for folate, none of the other nutrients that we analyzed alone or in conjunction with others influenced the risk of CRC. For folate, we observed that an intake of folate > 406.7 μg/d reduced the risk of CRC in >30% (OR: 0.67; 95% CI: 0.45, 0.99). This is in agreement with most prospective epidemiologic studies published so far (16, 17), although recently published studies raise the hypothesis whether a high folate intake in patients harboring premalignant lesions, ie, colorectal adenomas, might actually increase the risk of CRC (7, 18).

TABLE 1
Daily nutritional intake of participants[1]

	Cases (n = 196)	Controls (n = 200)	P	OR (95% CI)[2]
Vitamin B-6 (mg/d)	2.83 ± 1.06[3]	2.85 ± 0.98	NS	0.77 (0.50, 1.14)
Vitamin B-12 (μg/d)	14.5 ± 9.1	14.3 ± 8.23	NS	0.98 (0.66, 1.45)
Folate (μg /d)	401.6 ± 161.9	434.3 ± 161.3	0.02	0.67 (0.45, 0.99)
Glycine (mg/d)	5.39 ± 2.8	5.10 ± 2.34	NS	1.00 (0.68, 1.50)
Methionine (mg/d)	2.85 ± 1.28	2.78 ± 1.13	NS	0.99 (0.67, 1.50)
Serine (mg/d)	5.15 ± 2.20	5.14 ± 1.95	NS	0.89 (0.59, 1.30)
Alcohol (g/d)	25.17 ± 39.8	19.14 ± 43.42	NS	1.08 (0.73, 1.61)

[1] Differences in nutrients were determined with the Mann-Whitney test, adjusted for age, sex, and colorectal cancer history. OR, odds ratio.
[2] OR was determined with the values above or below the nutrient median intake. The cutoffs were as follows: vitamin B-6, 12.5 mg; vitamin B-12, 12.5 μg; folate, 406.7 μg; methionine, 2.58 mg; serine, 4.86 mg; glycine 4.68 mg; and alcohol, 3.4 g/d.
[3] $\bar{x}$ ± SD (all such values).

TABLE 2
Genotypes frequencies and associations with colorectal cancer risk[1]

	Cases (n = 196)	Controls (n = 200)	P[2]	OR (95% CI)[3]
	% (n)	% (n)		
MTHFR				
CC/CT	86.7 (170)	95.5 (191)	0.002	1
TT	13.3 (26)	4.5 (9)		3.01 (1.3, 6.7)
Allele C	67.3 (264)	68.8 (275)	NS	1
Allele T	32.7 (128)	31.2 (125)		1.07 (0.79, 1.44)
MTR				
AA/AG	98.0 (192)	98.0 (196)	NS	1
GG	2.0 (4)	20.0 (4)		0.77 (0.18, 3.3)
Allele A	84.8 (333)	84.9 (339)	NS	1
Allele G	15.1 (59)	16.7 (67)		0.90 (0.61, 1.32)
SHMT				
CC/CT	90.0 (177)	95.5 (191)	0.04	1
TT	10.0 (19)	4.5 (9)		2.6 (1.1, 5.9)
Allele C	71.4 (280)	73.5 (294)	NS	1
Allele T	28.6 (112)	26.5 (106)		1.11 (0.81, 1.52)

[1] MTHFR, methylene tetrahydrofolate reductase; MTR, 5,10-methylenetetrahydrofolate reductase; SHMT, serine hydroxymethyltransferase; OR, odds ratio.
[2] Determined with the chi-square test.
[3] Wild-type–heterozygote genotype was the reference category for OR calculation (multiple regression). OR was adjusted for age, sex, and colorectal cancer history.

However, we would like to strengthen that this increased risk of CRC is observed when pharmacologic doses of folic acid (the synthetic form of folate with greater availability) is used and not with dietary folate intake alone. In regard to the influence that genetic polymorphisms exert in the risk of CRC, the polymorphism C677T of the *MTHFR* gene is probably the most studied, but its relation with the risk of CRC is still unclear. The presence of this polymorphic variant (TT) was shown to reduce in vitro the enzyme activity to 30% (19), which may result in a significantly lower DNA methylation in TT carriers than in CC and CT subjects (20).

Contrary to most previously published studies, which report a 50% reduction in CRC risk in persons carrying the TT genotype, we report that the TT genotype was associated with an increased risk of CRC (OR: 3.01; 95% CI: 1.3, 6.7). However, a critical examination of previously published studies suggests that the protective effect of the TT genotype seems to largely depend on folate intake. Thus, among the first studies performed in Northern American countries, Chen et al (21) found that the TT genotype could actually increase the risk of CRC in men with high alcohol consumption (OR: 1.56; 95% CI: 0.65, 3.81). Alcohol is a known antagonist of folate metabolism and similarly to folate depletion; chronic alcohol abuse was shown to result in DNA hypomethylation (22). In a later study, Ma et al (23) showed that the TT genotype exhibited a protective effect in the risk of CRC but only in patients with a high folate intake. In men with low folate intake, the TT genotype was actually associated with an increased risk of developing CRC (OR: 1.33; 95% CI: 0.34, 5.17). Note that all the studies performed in North America since then (which unequivocally show a protective effect of the TT genotype) were performed after the mandatory fortification of enriched uncooked cereal grains with folic acid. Finally, we also have to consider that ≤35–50% of US adult citizens regularly consume a vitamin supplement, most of which provide 400 μg of folic acid per pill. All of the above considerations are possible explanations for the fact that since 1998-2000 all studies performed in the United States that examined the effect of C677T *MTHFR* polymorphism found a protective effect for the TT genotype because folate intake was in the high range for most if not all persons (23, 24). In the study published by Le Marchand et al (25) the researchers observed that total folate intake was between 500 and 600 μg/d, whereas in the present series we observed an intake ≈400 μg/d. This different intake is a plausible explanation for the different results obtained in studies coming from Europe (26–28), Mexico (29), or even Australia (30) where the TT genotype was actually found to be associated with an increased risk of CRC. In neither Europe nor Australia has fortification with folic acid been implemented; therefore, it is likely that folate status is substantially lower than in the countries were fortification has taken place. In further support of this hypothesis, when we examined the interaction between folate intake and *MTHFR* polymorphism, we observed that low folate intake was particularly deleterious in TT carriers (OR: 14.0; 95% CI: 1.8, 108.5). The reason for not having observed a protective effect of folate intake in TT carriers probably relates to the fact that in our population folate intake might not reach the amount required that would change TT genotype into a protector factor.

In the present study we also examined the effect of a recently described polymorphism in *MTR*, A2756G (31). Similar to our present study, Ma et al (5) and Marchand et al (25) observed that

TABLE 3
Odds ratio (OR) of colorectal cancer according to C677 *MTHFR* (methylene tetrahydrofolate reductase) genotype and diet[1]

	C677T *MTHFR*		P for interaction
	CC/CT	TT	
Vitamin B-6			
High	0.83 (0.55, 1.26)[2]	1.77 (0.67, 4.68)	NS
Low	1	7.22 (1.6, 32.6)	
Vitamin B-12			
High	1.01 (0.67, 1.53)	2.71 (0.9, 8.0)	NS
Low	1	3.95 (1.2, 12.4)	
Folate			
High	0.77 (0.51, 1.17)	1.50 (0.59,3.8)	0.05
Low	1	14.0 (1.8108.5)	
Glycine			
High	1.1 (0.7, 1.6)	2.0 (0.7,5.3)	NS
Low	1	8.2 (1.8, 36.9)	
Methionine			
High	1.1 (0.75, 1.76)	2.1 (0.8, 6.2)	NS
Low	1	5.9 (1.6, 1.3)	
Serine			
High	0.92 (0.6, 1.4)	1.79 (0.63, 5.14)	NS
Low	1	5.7 (1.6, 20.4)	
Alcohol			
High	1.1 (0.72, 1.6)	3.5 (1.1, 11.3)	NS
Low	1	3.2 (1.1, 9.5)	

[1] Combined genotype wild type–heterozygote (CC/CT) and low intake were the reference category for OR calculation (multiple regression). OR was adjusted for age, sex, and colorectal cancer history and determined with the values above or below the nutrient median intake. The cutoffs were as follows: vitamin B-6, 12.5 mg; vitamin B-12, 12.5 μg; folate, 406.7 μg; methionine, 2.58 mg; serine, 4.86 mg; glycine 4.68 mg, for alcohol, 3.4g/d.
[2] OR; 95% CI in parentheses (all such values).

MTR polymorphism was not associated with CRC risk, nor was there a significant interaction with any of the methyl-donor nutrients analyzed.

Finally, we also studied the C1420T1 *SHMT1* polymorphism. To our knowledge, there is only one study that examined the relation between *SHMT1* genotype and risk of CRC, and it did not observe any interaction (7). Recently, Van den Donk et al (32) studied the relation between this polymorphism, diet, and colorectal adenomas and concluded that the *SHMT1* C1420T polymorphism does not play a role in colorectal carcinogenesis. One hypothesis for this lack of association, which was postulated by Chen et al (8), is that SHMT2 (another isoform of the same enzyme) can also supply the one-carbon units required for cytosolic folate metabolism. In the present series we observed that this polymorphism was associated with an increased risk of developing CRC, although the strength of our observation is limited by the small number of participants homozygous for the variant ($n = 9$). Because the functional significance of this polymorphism is not yet known and because of the small numbers of homozygous participants, these results will need further confirmation.

In conclusion, in the present study we show that the C677T *MTHFR* polymorphism modifies the risk of developing CRC according to folate intake. To our knowledge, the influence of this genetic polymorphism was never examined in Southern European countries where pattern of folate and other methyl-donor nutrients, as well as alcohol, is certainly different from Northern Europe or American countries. Because mandatory folic acid fortification was never implemented in our country and vitamin supplements are not popular, it is believed that our population has a relatively low folate intake. This is a plausible explanation for the fact that, in our population, the TT genotype is associated with increased risk of developing CRC, particularly in participants with low folate intake (Table 3).

The author's responsibilities were as follows—CSG: design, subject evaluation, data collection, analysis, and manuscript preparation; BC: data collection, analysis, and manuscript preparation; SG: data collection; EC: analysis and manuscript preparation; PF: data collection; MB: data collection, manuscript preparation; CNL: data collection; MC: design, subject evaluation, data collection, analysis, and manuscript preparation. None of the authors had any financial or personal conflicts of interest.

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