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ORIGINAL ARTICLE

METHYLENETETRAHYDROFOLATE REDUCTASE C677T POLYMORPHISM AND RISK OF COLORECTAL CANCER IN THE MACEDONIAN POPULATION

Matevska N[1]**, Josifovski T[2]**, Kapedanovska A[1], Sterjev Z[1], Serafimoska Z[1], Panovski M[2], Jankulovski N[2], Petrusevska N[3], Angelovska B[3], Petrusevska G[4], Suturkova L[1], Dimovski AJ[1]*

***Corresponding Author:** Aleksandar J. Dimovski, Ph.D., Center for Biomolecular Sciences, Faculty of Pharmacy, University Ss Cyril and Methodius, Skopje 1000, Republic of Macedonia; Tel.: +389-2-3217 580; 3119 694; Fax: +389-2-3290 830; 3123 054; e-mail: adimovski@ff.ukim.edu.mk

ABSTRACT

Methylenetetrahydrofolate reductase (MTHFR) regulates the flow of folate groups between DNA synthesis and DNA methylation. A common C677T substitution (Ala222Val) in exon 4 of the MTHFR gene has been linked with the risk of colorectal cancer (CRC). To assess this risk in the Macedonian population, we conducted a case-control study of 413 randomly selected CRC patients and 185 controls without a clinical diagnosis of CRC. We found a statistically significant inverse association between the MTHFR T allele (35.35% for the patients and 41.35% for the controls) and the CRC risk [odds ratio (OR) 0.776; 95% confidence interval (95% CI) 0.603-0.997; $p = 0.047$). The prevalence of the MTHFR T allele is lower in patients with advanced CRC (Duke' s stage C and D) and with microsatellite instable tumors (MSI+), indicating the inverse association with the CRC aggressiveness and MSI status. This effect seems to be independent of gender, age of onset and localization. We concluded that the MTHFR 677T allele is more likely to have a protective effect on CRC development and progression in the Macedonian population.

Key words: Methylenetetrahydrofolate reductase (MTHFR); C677T polymorphism; Colorectal cancer (CRC)

INTRODUCTION

Methylenetetrahydrofolate reductase (MTHFR) plays a central role in folate metabolism, regulating the flow of folate groups between two important biosynthetic pathways: DNA synthesis and DNA methylation (Figure 1) [1-4]. Methylenetetrahydrofolate reductase catalyzes the irreversible reduction of 5,10-methylenetetrahydrolate (5,10-methyleneTHF) to 5-methyltetrahydrofolate (5-methylTHF), the main circulating form of folate in plasma and provides methyl groups for *de novo* synthesis of methionine, the precursor of S-adenosylmethionine (SAM). S-

1 Center for Biomolecular Sciences, Faculty of Pharmacy, University of Ss Cyril and Methodius, Skopje, Republic of Macedonia
2 Clinic for Abdominal Surgery, University of Ss Cyril and Methodius, Skopje, Republic of Macedonia
3 Institute of Radiotherapy and Oncology, University of Ss Cyril and Methodius, Skopje, Republic of Macedonia
4 Institute of Pathology, Faculty of Medicine, University Ss Cyril and Methodius, Skopje, Republic of Macedonia
** These two authors contributed equally to the study presented here and should both be considered as first authors.

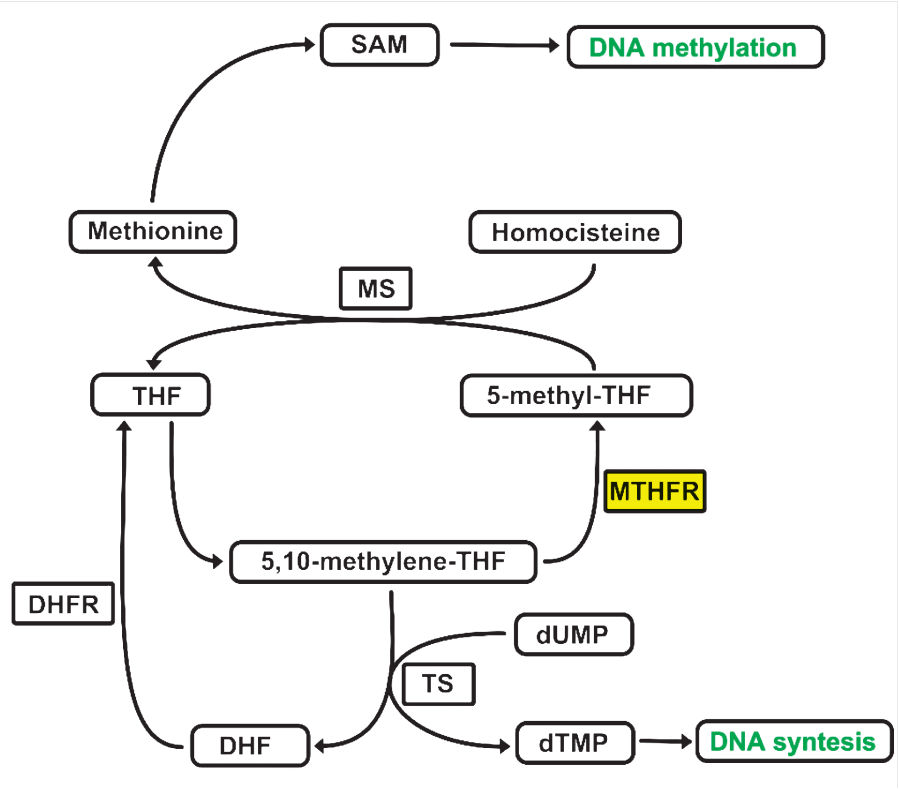

Figure 1. Schematic presentation of folate metabolism in relation to DNA methylation and thymidylate synthesis. THF: tetrahydrofolate; DHF: dihydrofolate; DHFR: dihydrofolate reductase; MS: methionine synthase; TS: thymidylate synthase; dUMP: deoxyuridine monophosphate (deoxyuridylate); dTMP: deoxythymidine monophosphate (deoxythymidylate); SAM: S-adenosylmethionine.

adenosylmethionine is the universal methyl-group donor for methylation of a wide variety of biological substrates, including DNA methylation, and may thereby contribute to carcinogenesis [5-9]. The substrate for MTHFR, 5,10-methyleneTHF, is an intracellular form of folate required for the conversion of deoxyuridylate (dUMP) to deoxythymidylate (dTMP) and is therefore vital for DNA synthesis. Depletion of this form of folate may produce deoxynucleotide pool imbalances, massive uracil incorporation into DNA and double-strand chromosome breaks, a feature commonly seen in colorectal cancer [10,11].

The MTHFR gene is located on chromosome 1p36.3. A common C677T substitution (Ala222Val) in exon 4 has been linked with the risk of numerous diseases, including cancer [12]. The variant enzyme is associated with a reduced enzyme activity and increased thermolability [12,13]. Individuals homozygous for the variant allele (677TT) have approximately 30%, whereas heterozygotes (677CT) have 65% of the normal enzyme activity. Compared with the wild-type, heterozygotes and TT homozygotes have lower plasma folate levels, raised homocysteine levels and reduced global DNA methylation in peripheral leucocytes [13-16].

Numerous studies have been made about the association between the C677T polymorphism and susceptibility to colorectal cancer (CRC). The majority point to a lower prevalence of CRC among individuals with the 677TT genotype [14,18-24], especially at high levels of folate intake [2,17]. However, some studies have implicated the C allele with increased CRC risk [25-27], while others have attributed no significant value to any allele of the C677T polymorphism [28,29]. Ethnicity, lifestyle, and pattern of diet may have introduced variability into different studies. To assess the MTHFR C677T polymorphism as a risk factor for CRC in the Macedonian population, we

conducted a case-control study of 413 randomly selected CRC patients and 185 controls without a clinical diagnosis of CRC.

MATERIALS AND METHODS

A total of 413 randomly selected patients with CRC and 185 control subjects from the Republic of Macedonia were recruited from the University Clinic of Abdominal Surgery and the Institute of Radiotherapy and Oncology in Skopje, between January 2006 and October 2008 (Table 1). Written informed consent was obtained from all participants of the study before the collection of the specimens. A questionnaire was used to elicit detailed information on demographic and clinical variables, prior disease history and family history of cancer. The average age of the patients was 60.27 ± 11.08 years. The control subjects consisted of 94 adults (average age 74.52 ± 10.46) and 91 newborns without any history of malignant disease. Since both control groups showed a similar genotype distribution, the results were integrated in further calculations.

Genomic DNA was isolated from peripheral blood and tumors using Proteinase K digestion/phenol-chloroform extraction and ethanol precipitation. The MTHFR C677T polymorphism (rs1801133) was genotyped by real-time polymerase chain reaction (PCR) (MxPro 3005P, Stratagene, La Jolla, CA, USA) using the TaqMan SNP genotyping assay according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA).

Descriptive comparisons [*i.e.,* means, standard deviation (SD), frequencies as percentages] of CRC patients and controls were conducted using a chi-square test for categorical variables and analysis of covariance for continuous variables. Logistic regression was used to calculate odds ratios (ORs) and corresponding 95% confidential intervals (95% CI). To examine separate and combined effects of the MTHFR genotype and certain risk factors, stratified analyses were conducted.

Table 1. General characteristics of the colorectal cancer patients and controls

	CRC Group: *n* (%)	Control Group[a]: *n* (%)
Total number of subjects	413	185
Gender:		
• males	238 (57.35)	81 (43.78)
• females	177 (42.65)	104 (56.22)
Age (years):		
• mean ± SD	60.27 ± 11.08	
• range	15-88	
Tumor site:		
• proximal	118 (28.57)	
• distal	295 (71.43)	
Tumor stage (Duke's):		
• A	33 (7.99)	
• B	181 (43.82)	
• C	163 (39.47)	
• D	36 (8.72)	
MSI status[b]:		
• MSI+	30 (11.81)	
• MSI–	224 (88.19)	

CRC: colorectal cancer; MSI: microsatellite instability.
[a] Ninety-one adults (mean ± SD: 74.52 ± 10.46; range 48-97) and 94 newborns.
[b] Determined for 254 subjects.

RESULTS

The characteristics of the study participants are presented in Table 1. Allele and genotype frequencies of MTHFR C677T polymorphism are presented in Table 2. No significant deviations from Hardy-Weinberg equilibrium were noted in either group. We found a statistically significant inverse association between the MTHFR T allele (35.35% for CRC patients and 41.35% for controls) and CRC risk (OR 0.776; 95% CI 0.603-0.997; $p = 0.047$). Prevalence of the variant allele (677T) within this population did not differ significantly from the prevalences reported in other Caucasian populations (30-35%) [30,31].

We observed a difference in overall genotype distribution between the CRC patients and controls (CC 42.61%; CT 44.07%; TT 13.32% for CRC patients and CC 35.13%; CT 47.03%; TT 17.84% for controls) even though it did not reach statistical significance ($p = 0.064$ TT; $p = 0.185$ CT; CC as reference). The MTHFR 677T allele was not associated with CRC either in a recessive (TT *vs.* CT + CC) or in a dominant (TT + CT *vs.* CC) model (OD 0.708; 95% CI 0.442-1.134; $p = 0.149$ and OD 0.729; 95% CI 0.729; $p = 0.085$ respectively), although there was a trend for significance towards the dominant model.

We stratified the subjects according to gender, age, and tumor stage, localization and microsatellite instability (MSI) status. Table 3 provides the ORs for the association between these variables and CRC risk (in all calculations the C allele was used as a reference). The results point towards a protective role of the MTHFR T allele in the progression of CRC, as shown with the lower prevalence of the T allele in patients with aggressive CRC (OD 0.682; 95% CI 0.484-0.961; $p = 0.028$ for Duke's C and OR 0.575; 95% CI 0.305-1.085; $p = 0.084$ for Duke's D). Although with borderline significance, we found a similar inverse association between the MTHFR 677T allele and MSI status (OD 0.561; 95% CI 0.308-1.020; $p = 0.055$).

Table 2. Association between *MTHFR* genotype and colorectal cancer risk

MTHFR Genotype	CRC Patients (*n*) (%)	Controls (*n*) (%)	Odds Ratios	95% Confidence Interval	*p* Value
Genotype Frequencies					
CC	**176 (42.61)**	**65 (35.13)**	1.00	Reference	
CT	**182 (44.07)**	**87 (47.03**	0.773	0.527–1.132	0.185
TT	**55 (13.32)**	**33 (17.84)**	0.616	0.367–1.032	0.064
CC and CT	358 (88.68)	152 (82.16)	1.00	Reference	
TT	55 (13.32)	33 (17.84)	0.708	0.442–1.134	0.149
CC	176 (42.61)	65 (35.13)	1.00	Reference	
CT and TT	237 (57.39)	120 (64.87)	0.729	0.509–1.025	0.805
Allele Frequencies					
C	534 (64.65)	217 (58.65)	1.00	Reference	
T	292 (35.35)	153 (41.35)	0.776	0.603–0.997	0.047

DISCUSSION

The results from our study suggest that the T allele is more likely to have a protective effect in

Table 3. Association between MTHFR and colorectal cancer risk after stratification by clinicopathological parameters

	Odds Ratios	95% Confidence Intervals	*p* Value
Gender:			
• males	0.835	0.580–1.203	0.333
• females	0.700	0.492–0.998	0.048
Age of onset:			
• > 60 years	0.812	0.604–1.092	0.168
• < 60 years	0.799	0.603–1.059	0.117
Tumor site:			
• proximal	0.828	0.592–1.159	0.270
• distal	0.849	0.651–1.108	0.228
Tumor stage (Duke's):			
• A	1.114	0.614–2.021	0.721
• B	0.830	0.599–1.150	0.263
• C	0.682	0.484–0.961	0.028
• D	0.575	0.305–1.085	0.084
MSI status[b]:			
• MSI+	0.561	0.308–1.020	0.055
• MSI–	0.843	0.636–1.117	0.234

CRC: colorectal cancer; MSI: microsatellite instability.
Odds ratio: C allele is used as reference.

CRC development in the Macedonian population. The prevalence of the MTHFR T allele was lower in patients with advanced CRC (Duke's stages C and D) and with microsatellite instable tumors (MSI+), indicating an inverse association with the CRC aggressiveness and MSI status. This effect seemed to be independent of gender, age of onset and localization since there was no difference after the stratification regarding these variables. Methylenetetrahydrofolate reductase regenerates the methionine from homocysteine, to maintain the supply of SAM for diverse methylation reactions of DNA, RNA, proteins and lipids. Total plasma homocysteine (tHcy) is a functional indicator of MTHFR activity and is correlated with the possession of the thermolabile form of the enzyme [32] and is inversely associated with DNA methylation [33]. DNA hypomethylation, particularly within promoter regions, could potentially reverse methyl-mediated silencing of oncogenes. Because of all of these, it could be expected that MTHFR 677TT is likely to increase the risk of CRC. Although some studies support this hypothesis [25-27], the majority of the studies, as well as two recent meta-analyses, have found a reduced risk of CRC associated with the MTHFR C677T homozygous variant (TT) genotype [14,18-24,34,35] that agree with our results (Figure 2). An alternative to the DNA hypomethylation hypothesis to account for the reduced risk associated with the C677T polymorphism has been proposed [18]. The higher activity of the wild-type enzyme may reduce the availability of 5,10-methyleneTHF for synthesis of dTMP from dUMP. Low erythrocyte folate levels (<140 ng/mL) were associated with increased frequency of DNA double-strand breaks as a consequence of the mis-incorporation of uracil.[36] The TT genotype would slow down the conversion of 5,10-methyleneTHF to 5-methylTHF and close

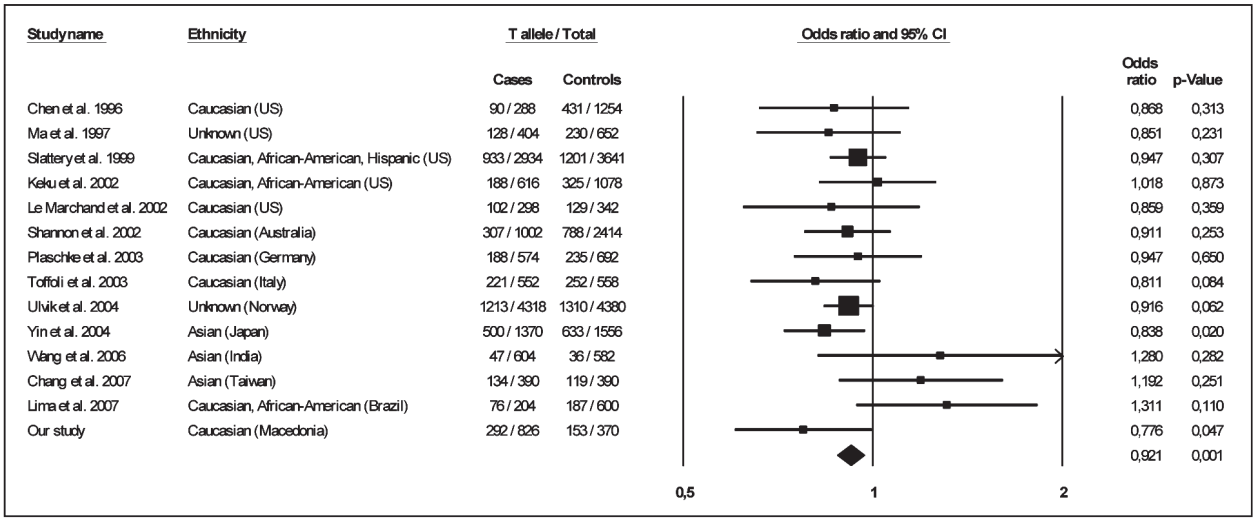

Figure 2. Overall meta analysis for the C677T polymorphism (T *vs.* C allele) in colorectal cancer. Point estimates of the odds ratios for each study and the accompanying 95% confidence interval values obtained with a fixed effects model are shown.

this avenue for DNA damage [31]. Therefore, the MTHFR C677T polymorphism would modify the CRC risk and CRC advancement by influencing the availability of 5,10-methyleneTHF for DNA synthesis and repair rather than through DNA methylation. The conflicting results from different studies (Figure 2), may result from dissimilarity in lifestyle and dietary habits in different populations (*e.g.,* diet rich or low in folate, alcohol consumption), and from polymorphisms in genes encoding for other enzymes (Dihydrofolate reductase; Methionine synthase; Thymidylate synthase or others), interconnected in folate metabolism.

The inverse association between the MTHFR 677T allele and the MSI+ that we observed, can be explained by the DNA hypomethylation hypothesis. The lower enzyme activity in homozygotes for the variant allele (677TT) could lead to reduced epigenetic silencing of the mismatch repair (MMR) genes, this being the main reason for MSI in sporadic CRC.

In conclusion, our results suggest that the MTHFR 677T allele is more likely to have a protective effect in CRC development and progression in the Macedonian population, and support previous findings of an inverse association of the MTHFR 677T allele with CRC. Knowledge of the interaction between folate intake and the MTHFR polymorphisms could be useful in elucidating the role of the MTHFR polymorphisms in colorectal carcinogenesis.

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