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Lack of significant association between MTHFR gene C677T polymorphism and colorectal cancer in the Azerbaijani population

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ABSTRACT. We evaluated methylenetetrahydrofolate reductase gene *C677T* polymorphisms in patients with colorectal cancer in a population-based case-control study in the Azerbaijan population. Genomic DNA was isolated from blood samples taken from 155 patients with colorectal cancer and 155 healthy individuals. The MTHFR gene C677T polymorphism was detected on agarose gel by PCR-RFLP. The frequencies of the *CC*, *CT*, and *TT* genotypes of *MTHFR* (*C677T*) were 54, 37, and 9% in the patients with colorectal cancer and 65, 29, and 6% in the healthy control, respectively. Heterozygote *CT* (OR = 1.422, 95%CI = 0.883–2.289, P = 0.147) and homozygous mutant *TT* (OR = 1.440, 95% CI = 0.619–3.348, P = 0.395) genotypes were more frequent in colorectal cancer patients compared to controls. No significant associations were observed between genotype and allele frequency of the *MTHFR* gene and the risk of colorectal cancer. Our findings suggested that *MTHFR C677T* polymorphism might not be associated with the

overall risk of colorectal cancer in an Azerbaijani population. However, these conclusions need to be validated in a larger cohort study.

Key words: *C677T*; Colorectal cancer; Folate metabolism; Methylation; *MTHFR*; Polymorphisms

INTRODUCTION

Colorectal cancer (CRC), a heterogeneous disease caused by genetic and environmental interactions, is the third most common malignancy and the second leading cause of cancer-related deaths worldwide (Bray et al., 2018). Chromosomal abnormalities (Pino & Chung, 2010), inactivation of tumor suppressor genes (Hardiman, 2018), oncogene activation (Seiden-Long et al., 2006), microsatellite instability (MSI) (Vilar & Gruber, 2010), DNA methylation (Hinoue et al., 2012), abnormalities in DNA repair genes, and single nucleotide polymorphisms (SNPs) (Lord & Ashworth, 2012) are involved in the development of CRC.

Studies have demonstrated that diets with a high intake of red meat (Larsson and Wolk, 2006; Zhao et al., 2017), alcohol consumption (Jayasekara et al., 2016) and smoking (Botteri et al., 2008; Liang et al., 2009), sedentary lifestyle (Huxley et al., 2009), obesity (Moghaddam et al., 2007), and low consumption of vegetables, fruit, and dietary fiber (Terry et al., 2001) are associated with an increased CRC risk. It has also been reported that high consumption of fruits and vegetables, fish, and certain nutrients such as selenium, calcium, vitamin D, and folate may have a protective effect against CRC (Azeem et al., 2015). Altered folate metabolism is associated with abnormal DNA methylation (hypo- and hypermethylation) of tumor suppressor genes, which plays a role in colon carcinogenesis (Jokić et al., 2011; Wu et al., 2015).

Methylenetetrahydrofolate reductase (MTHFR) is an important enzyme in folate metabolism that irreversibly converts 5,10 methylenetetrahydrofolate (5,10-methylene-THF) to 5-methyl tetrahydrofolate (El Awady et al., 2009); it should be noted that 5-methyl-THF is the most stable form of MTHFR that provides methyl group for DNA methylation and methionine synthesis (Fernández-Peralta et al., 2010; Yousef et al., 2013). MTHFR regulates circulating folate and methionine levels and prevents the accumulation of homocysteine in the blood (Lucock, 2004).

The human *MTHFR* gene is localized on chromosome 1p36.3 and encodes the *MTHFR* enzyme that consists of 656 amino acids (Awwad et al., 2015). The single nucleotide polymorphism (*C→T*, alanine to valine) within codon 222 in exon 4 (*C677T*) of the *MTHFR* gene reduces enzyme activity, resulting in a decrease in folate levels in the blood (Li et al., 2011). Depending on whether the *C677T* genotype is heterozygous or homozygous, it has been reported that the enzyme activity in individuals is 30% and 65%, respectively (Frosst et al., 1995; Torre et al., 2014). The low enzymatic activity of the *MTHFR C677T* variant is associated with global DNA hypomethylation in peripheral leucocytes, which may induce genomic instability; this can also affect the expression of oncogenes or tumor suppressor genes (Cui et al., 2010). Several studies indicated that polymorphism of *MTHFR C677T* was associated with an increased risk of CRC (Delgado-Enciso et al., 2001; Shannon et al., 2002; Hubner and Houlston, 2007). However, other studies reported that the homozygous variant (*TT*) of the *MTHFR* gene decreased the risk of

CRC (Le Marchand et al., 2005). On the other hand, other studies have observed no relationship between the *MTHFR C677T* genotype and the genetic susceptibility of CRC (Zeybek et al., 2007; Derwinger et al., 2009).

In the current case-control study, we focused on the Azerbaijan population to evaluate a potential association of the *MTHFR C677T* gene polymorphism with CRC risk.

MATERIAL AND METHODS

Subjects

The study included 155 CRC patients and a control group of 155 healthy individuals Blood samples from patients and controls were collected at the Scientific Center of Surgery (Baku, Azerbaijan) between 2017-2020. In addition, age and gender-matched healthy individuals were randomly selected from cancer-free individuals and included study as control groups who get colonoscopies during a routine check-up in the same center. The ethics committee of the Scientific Center of Surgery (Baku, Azerbaijan) approved this research. Written informed consent from all patients and controls was obtained. All individuals agreed to participate in our study. The patient group consisted of 92 men and 63 women (25-85 years old), whereas the control group consisted of 76 men and 79 women (28-86 years old). The patients were diagnosed histopathologically with CRC. Physically healthy and cancer-free volunteers were included in the control group. Venous blood samples were collected from patients and controls in tubes with ethylenediaminetetraacetic acid (EDTA) and then transferred to the Laboratory of Human Genetics of the Institute of Genetic Resources of the Azerbaijan National Academy of Sciences for DNA extraction.

DNA extraction and *MTHFR C677T (rs1801133)* genotyping

DNA extraction from blood samples was performed according to the QIAamp DNA Blood Midi Kit protocol. DNA samples were stored at - 20°C until required for polymerase chain reaction (PCR) amplification. The quantity and quality of DNA were determined on a NanoDrop™ 2000/2000c spectrophotometer (ThermoFisher Scientific, USA).

MTHFR C677T (*rs1801133*) genotyping was performed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) methods. PCR reactions were performed using *5'-TCCCTGTGGTCTCTTCATCC-3*' (forward) and *5'-ACTCAGCACTCCACCCAGAG-3'* (reverse) primers for *MTHFR C677T* polymorphism (Table 1).

Table 1. Restriction enzyme and primer sequencing for *MTHFR C677T* polymorphism.

Gene	Primers	PCR product	Restriction Enzyme	Genotypes
MTHFR C677T	F:5'T*CC*CTGTGGTCTCTTCAT*CC*3' R:5'TGAGG*CC*CGAAACA*CC*CGTA3'	360 bp	HinfI	*CC*: 360 bp *CT*: 360 bp, 210 bp, 150bp TT: 210 bp, 150 bp

PCR was carried out in a total volume of 50 µl, containing 100 ng genomic DNA, 0.02 U Taq DNA polymerase (Solis BioDyne, Tartu, Estonia), 10x Taq DNA polymerase buffer, 2 mM $MgCl_2$, and 0.25 mM dNTP, with 100 ng of each primer. Following initial denaturation at 95°C for 5 minutes, 35 amplification cycles of 95°C for 5 minutes, annealing temperature for 58°C for 1 minute, and elongation at 72°C for 2 minutes were performed. PCR amplicons were then visualized on a 1% agarose gel under a Gel Doc™ XR + Imager (Bio-Rad) system. The PCR products were then processed by Restriction Fragment Length Polymorphism (RFLP) using the *HinfI* (NEB, New England Biolabs) restriction enzyme (incubation 37°C) and the results were analyzed on 2% agarose gel. As a result of RFLP, the wild-type allele remained intact (360 bp), heterozygous forms were fragmented into 360 bp, 210 bp, and 180 bp and homozygous mutant alleles into 210 bp and 180 bp (Figure 1).

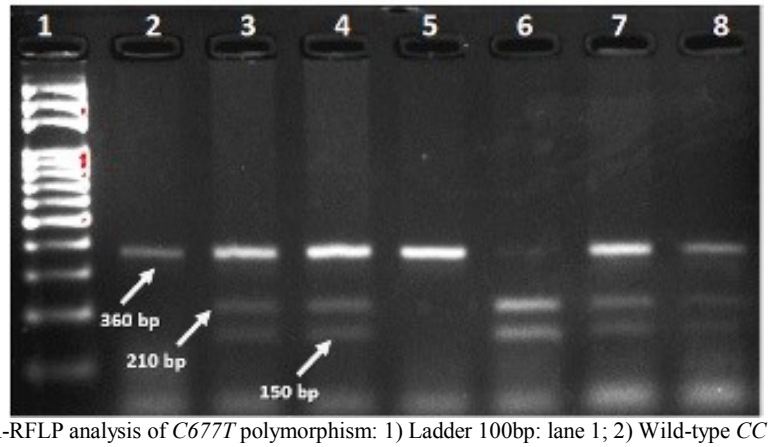

Figure 1. PCR-RFLP analysis of *C677T* polymorphism: 1) Ladder 100bp: lane 1; 2) Wild-type *CC* (360bp): lanes 2 and 5; 3) Homozygote *TT* (210 and 150bp): lane 6; 4) Heterozygote *CT* (360, 210, and 150bp): lanes 3, 4, 7, and 8.

Statistics

Statistical analysis was performed using the SPSS (Chicago, IL, USA) software version 22. The observed frequency of genotypes in CRC was compared to that of the controls using chi-square or Fisher's exact tests. The relative risk for the *MTHFR C677T* polymorphism was calculated as an odds ratio (OR) with 95% confidence interval (CI), using individuals who were homozygous wild type as the reference group. $P < 0.05$ was considered statistically significant.

RESULTS

A total of 155 CRC patients and 155 control subjects were included in this study. The demographic characteristics of the patients and controls are shown in Table 2. CRC patients comprised 92 (59.4%) males and 63 (40.6%) females and the control subjects consisted of 76 (49%) males and 79 (51%) females. The age range was 25–85 (mean 63.3 ± 10.84) in patients and 28–86 (mean 63.9 ± 12.57) in controls.

Table 2. Characteristics of the colorectal cancer patients and healthy controls.

	Patients N=155 (%)	**Healthy Control N=155 (%)**
Gender		
Male	92 (59.4)	76 (49%)
Female	63 (40.6)	79 (51%)
Age		
Range	25-85	28-86
Mean ±SD	63.3±10.84	63.9±12.57
Tumor Grade		
G1	13 (8.4)	
G2	102 (65.8)	
G3	40 (25.8)	
Tumor Stage		
T1	3 (1.9)	
T2	17 (11)	
T3	123 (79.4)	
T4	12 (7.7)	

CRC patients were evaluated pathohistologically and tumor grade and stage were determined. Thirteen of the patients had G1, 102 had G2, and 40 had G3 tumor grade. In addition, T1 tumor stage was determined in 3 patients, T2 in 17 patients, T3 in 123 patients, and T4 in 12 patients (Table 2). Heterozygous CT and mutant TT genotypes were observed more frequently in tumor grade G2 and tumor stage T3. There was no statistically significant difference between C677T genotypes and tumor stage/grade (Table 3).

Table 3. Association of MTHFR C677T with colorectal cancer grade and stage.

Histological Grade	***CC*** **n (%)**	***CT*** **n (%)**	***TT*** **n (%)**	**P value**
G1	8 (9.5)	4 (7)	1 (7.1)	
G2	52 (61.9)	41 (72)	9 (64.3)	0.800
G3	24 (28.6)	12 (21)	4 (28.6)	
Tumor Stage				
T1	2 (2.4)	0	1 (7.1)	
T2	11 (13.1)	4 (7)	2 (14.3)	0.199
T3	64 (76.2)	48 (84.2)	11 (78.6)	
T4	7 (8.3)	5 (8.8)	0	

The genotype and allele frequency of the MTHFR C677T polymorphism and their adjusted OR and 95% CIs were calculated (Table 4). The MTHFR C677T frequencies of CC, CT, and TT genotypes were found in 54%, 37%, and 9% in patients with CRC and 65%, 29%, and 6% in the controls, respectively. The frequency of wild-type CC genotype was higher in the control group, whereas the incidence of heterozygous CT and homozygous mutant TT genotype was higher in the patient group compared to the control group. However, this difference did not reach statistical significance.

Heterozygous CT genotype was found to be more frequent in the CRC patients than control subjects. However, association was not statistically significant (P = 0.147). In addition, the frequency of mutant homozygous TT genotype was higher in patients with

CRC compared to healthy controls. The hazard ratio of the MTHFR TT genotype in patients with CRC was elevated; however there was no significant difference between CRC patients and controls.

Table 4. Genotype and allele frequencies of MTHFR C677T and the risk of colorectal cancer.

	Patients N=155 (%)	Healthy Controls N=155 (%)	OR (95%CI)	P value
Genotypes				
CC	84 (54)	100 (65)	Reference	1.00
CT	57 (37)	45 (29)	1.422 (0.883-2.289)	0.147
TT	14 (9)	10 (6)	1.440 (0.619-3.348)	0.395
CC vs *CT+TT*	84 vs 71	100 vs 55	1.513 (0.974-2.425)	0.064
CC+CT vs *TT*	141 vs 14	145 vs 10	1.440 (0.619-3.348)	0.395
Allele frequency				
C	225 (72.6)	245 (79)	Reference	1.00
T	85 (27.4)	65 (21)	1.424 (0.983-2.062)	0.061

P value, OR and 95% CI for the dominant model (CC vs CT+TT) were 0.064, 1.513 and 0.974-2.425, and 0.395, 1.440 and 0.619-3.348 was calculated for the recessive model (CC+CT vs TT). Moreover, the C allele and T allele frequency of MTHFR C677T polymorphism in the CRC patients were 72.6% and 27.4%, respectively. The normal C allele frequencies of MTHFR at position 677 were 79% in controls and the mutant T allele frequencies were 21%. Mutant T allele increased the risk of CRC, but no statistical significance was identified (P = 0.061).

DISCUSSION

Folate metabolism is important for maintaining genome stability due to its role in DNA synthesis, repair, and methylation (Fowler, 2005; Levin and Varga, 2016). The *C677T* (rs1801133) polymorphism in exon 4 of the *MTHFR* gene replaces an alanine to valine amino acid in a codon 222 (Haghighi et al., 2009). This change affects the active site of the enzyme and reduces enzyme activity. In the present study, we investigated the associations between *MTHFR* gene polymorphisms and CRC risk. In our study, no statistical difference was observed between the genotype and allele frequency of the *MTHFR* gene and the risk of CRC.

Several studies have reported a controversial association between *MTHFR C677T* gene polymorphism and CRC risk among different populations. Sheng et al. found that MTHFR C667T polymorphism contributed to a lower colorectal cancer risk in the general population based on meta-analyses of 61 case-control studies (Sheng et al., 2012). This group performed a subgroup analysis by ethnicity and found a correlation between the T allele of MTHFR C667T and colorectal cancer risk in Asians. The MTHFR 677TT genotype was found to be associated with colorectal cancer inversely associated with folate intake and low alcohol consumption according to a multiethnic cohort study conducted by Taioli et al. (Taioli et al., 2009).

TT genotype was found to be associated with an increased risk for CRC in the Portuguese population (Guerreiro et al., 2008). However, no relationship was reported in the Turkish population (Zeybek et al., 2007). In the meta-analysis performed in a total of 9,143

CRC cases and 11,357 controls, no relationship was observed between the *MTHFR* gene *C677T* polymorphism and the CRC risk in the Asian population (Rai, 2015). However, another meta-analysis study in China suggested that *MTHFR C677T* polymorphism was associated with CRC susceptibility (Xu et al., 2017).

Yang et al. showed with meta-analyses that there was an obvious association of the *MTHFR 677T* allele with decreased risk (OR = 0.91, 95% CI = 0.85–0.98, P = 0.011) of CRC (Yang et al., 2012). Similarly, there was a statistically significant decrease in CRC risk (OR = 0.81, 95% CI = 0.63–1.0, P = 0.10) in the Canadian population for the *C677T* polymorphism (Levine et al., 2010). A meta-analysis of 25 populations indicated that individuals carrying the *677TT* genotype were at moderately reduced CRC risk (Hubner et al., 2007). The *677T* allele was reported to have shown a small but significant protective effect against CRC risk (Huang et al.,2007). On the other hand, *677TT* was reported to be associated with an increased CRC risk in older populations (Shannon et al., 2002).

Ryan et al. Reported that *MTHFR CT* heterozygotes had a significantly increased risk of developing CRC (OR = 1.86, 95% CI = 1.3–2.7, P < 0.05), and no increased cancer risk was observed in *TT* homozygotes in the Northern European population (Ryan et al., 2001). A study observed a decreased risk of colon cancer in individuals with *677CC* genotypes (Haghighi et al., 2009). There are various studies where no association was observed between *677TT* and colon cancer risk and these studies are in a*CC*ordance with our results (Keku et al., 2002; Komlósi et al., 2010).

We also calculated OR and 95% CI for the dominant (*CC* vs *CT* + *TT*) and recessive (*CC* + *CT* vs *TT*) model in our study. However, there was no obvious association between recessive and dominant genetic comparison models and CRC. Similar results were discovered in a meta-analysis study based on the Asian population (Rai, 2015). Another study showed that in the subgroup analysis, according to ethnicity, the OR was not significant in Caucasians and East Asians; the dominant and recessive models performed no significant associations overall in either Caucasians or East Asians (Zintzaras et al., 2009). On the contrary, some studies have found positive associations in both models (Lin et al., 2018).

In addition, we compared the patients taking into account the tumor grade, stage, and genotypes. In this comparison, no significant difference was identified. Hubner et al. was reported that the *MTHFR C677T* genotype was associated with the CRC tumor stage (Hubner et al., 2007). One study demonstrated that there was no significant association between *MTHFR* genotype and CRC stage (Ryan et al., 2001). No association was found between *MTHFR C677T* polymorphism and tumor grade and stage in either the Spanish population (Fernández-Peralta et al., 2010) or the northeast China population (Zhu et al., 2013).

In conclusion, the *C677T* polymorphism of the *MTHFR* gene in CRC patients in an Azerbaijani population was evaluated in this study for the first time and we found no convincing evidence that *MTHFR* gene *677CT* and *677TT* polymorphism is associated with increased risk of CRC in the Azerbaijani population. Our study showed no obvious association between recessive and dominant genetic comparison models and CRC. There was also no significant association between genotypes of *MTHFR C677T* and the tumor grade and stage. However, these correlations need to evaluated in a larger cohort study in order to be conclusive.

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AUTHORS CONTRIBUTIONS

Study design: Vugar Yagublu, Hazi Aslanov, and Bayram Bayramov; Data Collection: Asiman Hadizade, and Narmina Alakbarova; Data Analysis: Shalala Abdulrahimli, Bayram Bayramov; Manuscript preparation: Shalala Abdulrahimli, Vugar Yagublu, Christoph Reissfelder, Rauf Agaev, Hazi Aslanov, and Bayram Bayramov.

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DATA ACCESSIBILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon request.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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