RESEARCH ARTICLE

Investigation of the DNMT3B –579 G>T Promoter Polymorphism in Patients with Colorectal Cancer in an Azerbaijani Population

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Abstract

Objective: The main aim of the present study was to determine the clinical significance of the DNA methyltransferase 3B (*DNMT3B*) gene –579 G>T polymorphism in colorectal cancer (CRC) patients. **Methods:** A total of 140 patients with CRC and 164 healthy individuals were included in the study. According to the manufacturer's instructions, DNA was isolated from blood, and genotypes were determined on agarose gel by the PCR-RFLP method. Genotype confirmation was performed using Sanger sequencing in randomly selected samples. **Results:** When comparing the case and control groups, heterozygous GT (OR=0.53; 95% CI=0.32–0.88), under the dominant model (OR=0.53; 95% CI=0.33–0.87), and the mutant T allele (OR=0.71; 95% CI=0.51–0.98) were statistically associated with a reduced risk of CRC. However, when the age, pathological tumor grade and stage, smoking habit, and alcohol consumption were compared, no significant relationship was determined (P>0.05). Furthermore, among males, heterozygous GT was associated with a reduced risk of CRC (OR=0.40; 95% CI=0.19–0.84). **Conclusion:** Our study highlighted that the –579 G>T polymorphism of the *DNMT3B* gene plays a protective role against CRC development.

Keywords: DNMT3B- polymorphism- DNA methylation- colorectal cancer

Introduction

Colorectal cancer (CRC) is the fourth most common cancer worldwide, and its incidence is rapidly increasing in developed countries (Ferlay et al., 2015; George et al., 2018; Siegel et al., 2019). In addition, the number of new cases and deaths are predicted to reach 2.2 and 1.1 million by 2030, respectively (Ogunwobi et al., 2020). Studies have demonstrated that certain risk factors, such as carcinogenic agents, unhealthy diet, smoking, and heavy drinking, might increase the risk of CRC (Schweiger et al., 2013; Ma et al., 2018). Additionally, genetic and epigenetic modifications in oncogenes and tumor suppressor genes have been observed to play an essential role in the molecular pathogenesis of diseases (Muller et al., 2016). However, global DNA hypomethylation and gene-specific hypermethylation are possible inactivation mechanisms for tumor suppressor genes (Pan et al., 2018). DNA methylation is catalyzed by the DNA methyltransferase (DNMT) enzyme families (Morgan et al., 2018). The DNMT3B enzyme performs de novo methylation, which is necessary to establish methylation during development and genomic imprinting (Lyko, 2018;

Yagi et al., 2020).

The *DNMT3B* gene is located on chromosome 20q11.2 and consists of 23 exons and 22 introns (Lan et al., 2010; Ahmadi et al., 2018). The single nucleotide polymorphisms (SNPs) of the *DNMT3B* gene can significantly alter the DNA methylation activity of DNMT3B (Fan et al., 2008a; Ezzikouri et al., 2009; Lao et al., 2013). 149 C>T (rs2424913) and –579 G>T (rs1569686) occurring in the promoter region of the DNMT3B gene are the most common polymorphisms, and they have been investigated in various diseases (Guo et al., 2010; Hu et al., 2010; Chen et al., 2017). The DNMT3B –579 G>T polymorphism has been reported to be associated with a variety of tumors, including colon cancer, head and neck cancer, lung cancer, hepatocellular carcinoma, and acute myeloid leukemia (Khorshied and El-Ghamrawy, 2012; Liu et al., 2012; Zhang and Xu, 2017). In this study, we have evaluated the association between the DNMT3B –579 G>T polymorphism and the risk of CRC in the Azerbaijani population, which no other study has reported, to the best of our knowledge.

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Materials and Methods

Study population

This study included 140 CRC patients and 164 healthy controls. The patients included in the study were individuals with histologically confirmed cancer diagnoses and treated at the specialized gastrointestinal units of Scientific Center of Surgery named M.Topchubashov and the Educational-Surgical Clinic of Azerbaijan Medical University between 2017-2020. Age and gender-matched control subjects were randomly selected from healthy, cancer-free individuals, who got colonoscopies within the colon cancer screening program during the same period. Information on individuals' smoking habits and alcohol use were obtained through a questionnaire. Blood samples were collected in EDTA tubes, and DNA extraction was performed afterward. The scientific committee approved this study by the Genetic Resources Institute of ANAS.

DNA isolation and genotyping

Genomic DNA isolation from peripheral blood samples was performed using the QIAamp DNA Blood Mini kit (Qiagen, Germany), following the manufacturer's protocol. The *DNMT3B* gene −579 G>T polymorphism was detected by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. PCR reactions were performed in a total of 25 μl containing 100 ng of genomic DNA, 10 μM (10 pmol/μL) of both sense and antisense primer (sense: 5′-GGGGGCCTGGAGGTCTCATTAT-3′; antisense: 5′-ACGGATGGGTTGGCAGGCTATA-3′), 2.5 mM MgCl2, 2.5 μl FIREPol® 10x Buffer B (0.8 M Tris-HCl, 0.2 M (NH4)2SO4, 0.2% w/v Tween-20), 0.25 mM dNTP (Solis BioDyne, Tartu, Estonia), and 0.05 units of Taq DNA polymerase (Solis BioDyne, Tartu, Estonia). The cycling conditions for the PCR consisted of an initial denaturation step at 95°C for 5 mins, followed by 35 cycles at 95°C for the 30s, 60°C for 45s, 72°C for 60s, and a final extension at 72°C for 10 mins. The 343 bp PCR product was digested with the enzyme PvuII (New England Biolabs, NEB) for 3 h at 37°C and then separated on a 3% agarose gel, and the RFLP bands were visualized under ultraviolet (UV) light with ethidium bromide staining (Figure 1). Randomly selected PCR products were confirmed using direct sequencing, and the results were also 100% concordant (Figure 2).

Statistical analysis

The relationship between the genotypes and allele frequencies and the clinicopathologic parameters, age and sex characteristics, alcohol consumption, and smoking status were analyzed using Pearson's chi-squared test (χ^2) or Fischer's exact test. The association between the DNMT3B −579 G>T polymorphism and CRC cases were determined by calculating the odds ratios (ORs) and 95% confidence intervals (95% CIs). P<0.05 value was considered statistically significant. All statistical analyses were performed using the software package SPSS, version 15.0 (SPSS Inc., Chicago, IL).

Results

The demographic characteristics of the CRC cases and controls were summarized in Table 1. The mean age of the patients and controls was 62±10.2 years and 61.78±11.3 years, respectively. While 85 of the total patient group were male and 55 were female, 71 of the controls were male, and 93 were female. There was no significant difference in the study groups' age factor, smoking status, and alcohol consumption. However, no CRC risk was observed between the GT and TT genotypes and among individuals under and over 61 (Table 2). On the contrary, among males, the heterozygous GT genotype was found to be associated with a significantly reduced risk of CRC (OR=0.40; 95% CI=0.19–0.84). The frequency of the DNMT3B promoter −579 G>T polymorphism in cancer cases and controls is shown in Table 3. The frequency of GG, GT, and TT genotypes in patients with CRC were 40.7%, 42.9%, and 16.4%, respectively while it was 26.8%, 53.7%, and 19.5%, respectively, among healthy controls. In the present study, we found that the GT genotype was significantly associated with a decreased risk of CRC (OR=0.53; 95% CI=0.32–0.88). When the homozygous GG genotype of the *DNMT3B* gene was used as a reference, there was no significant relationship for the mutant TT genotype distribution (OR=0.56; 95% CI=0.29–1.08). Furthermore, a significantly decreased risk of CRC was observed under the dominant model

Table 1. Clinic and Demographic Characteristics of Study Groups

	Patients N=140 (%)	Healthy Control N=164 (%)	P value
Gender			
Male	85 (60.8)	71 (43.3)	0.002
Female	55 (39.2)	93 (56.7)	
Age			
Range	24-84	32-82	
Mean ±SD	62±10.2	61±11.3	
Tumor Grade			
G1	12 (8.6)		
G2	89 (63.5)		
G3	39 (27.9)		
Tumor Stage			
T1	3 (2.1)		
T2	13 (9.3)		
T3	114 (81.5)		
T4	10 (7.1)		
Smoking using			
Smokers	48 (34.3)	55 (33.5)	0.942
Non-smokers	84 (60)	98 (59.8)	
Unknown	8 (5.7)	11 (6.7)	
Alcohol drinking			
User	45 (32.1)	59 (36)	0.41
Non-user	87 (62.2)	93 (56.6)	
Unknown	8 (5.7)	12 (7.4)	

Table 2. Distribution of Genotypes According to Age and Gender of Study Groups

Genotypes	Cases	Controls	OR (95%CI)	P value
Male	N=85 (%)	N=71 (%)		
GG	35 (41.2)	16 (22.5)	1	-
GT	37 (43.6)	42 (59.2)	0.40 (0.19-0.84)	0.015
TT	13 (15.2)	13 (18.3)	0.46 (0.17-1.21)	0.111
Female	N=55 (%)	N=93 (%)		
GG	22 (40)	28 (30.1)	1	-
GT	23 (41.9)	46 (49.4)	0.64 (0.30-1.35)	0.236
TT	10 (18.1)	19 (20.5)	0.67 (0.26-1.73)	0.406
Age	N=58 (%)	N=79 (%)		
≤61				
GG	26 (44.8)	23 (29.1)	1	-
GT	24 (41.4)	45 (56.9)	0.47 (0.22-0.99)	0.148
TT	8 (13.8)	11 (14)	0.64 (0.22-1.86)	0.417
>61	N=82 (%)	N=85 (%)		
GG	31 (37.8)	21 (24.7)	1	1
GT	36 (43.9)	43 (50.6)	0.57 (0.28-1.15)	0.116
TT	15 (18.3)	21 (24.7)	0.48 (0.20-1.15)	0.097

Table 3. Genotypic and Allelic Frequencies of *DNMT3B* Gene

	Cases N=140 (%)	Controls N=164 (%)	OR (95% CI)	P value
Genotype				
GG	57 (40.7)	44 (26.8)	1	
GT	60 (42.9)	88 (53.7)	0.53 (0.32-0.88)	0.014
TT	23 (16.4)	32 (19.5)	0.56 (0.29-1.08)	0.081
Dominant model				
GG	57 (40.7)	44 (26.8)	1	-
GT+TT	83 (59.3)	120 (73.2)	0.53 (0.33-0.87)	0.01
Recessive model				
GG+GT	117 (83.6)	132 (80.5)	1	-
TT	23 (16.4)	32 (19.5)	0.81 (0.45-1.46)	0.486
Allele				
G	174 (62.1)	176 (53.7)	1	
T	106 (37.9)	152 (46.3)	0.71 (0.51-0.98)	0.035

Table 4. Tumor Grading and Staging

	GG, N (%)	GT, N (%)	TT, N (%)	P value
Tumor grade				
G1	6 (50)	5 (41.7)	1 (8.3)	0.362
G2	38 (42.7)	34 (38.2)	17 (19.1)	
G3	13 (33.3)	21 (53.9)	5 (12.8)	
Tumor stage				
T1	1 (33.3)	1 (33.3)	1 (33.3)	0.766
T2	6 (46.2)	4 (30.8)	3 (23)	0.766
T3	46 (40.4)	51 (44.7)	17 (14.9)	
T4	4 (40)	4 (40)	2 (20)	

Table 5. Association of Smoking and Alcohol Risk with *DNMT3B* Genotype Distribution among CRC Patients (Patients Only)

Genotypes	Smokers	Non-smokers	OR (95%CI)	P value
	N=48, (%)	N=84, (%)		
GG	22 (45.8)	34 (40.5)	1	-
GT	21 (43.8)	34 (40.5)	0.96 (0.45-2.05)	0.905
TT	5 (10.4)	16 (19)	0.48 (0.16-1.51)	0.205
	Alcohol drinkers	Non-drinkers		
	N=45, (%)	N=87, (%)		
GG	20 (44.4)	36 (41.3)	1	-
GT	20 (44.4)	35 (40.2)	1.02 (0.47-2.23)	0.943
TT	5 (11.2)	16 (18.5)	0.56 (0.18-1.77)	0.32

(OR=0.53; 95% CI=0.33–0.87), whereas there was a no significant association for CRC the recessive model (OR=0.81; 95% CI=0.45–1.46). The mutant T allele was associated with a significantly decreased risk of CRC (OR=0.71; 95% CI=0.51–0.98). Table 4 shows the genetic distribution of the DNMT3B genotypes according to tumor grades and stages. The DNMT3B −579 G>T polymorphism was not related to cancer risk at any stage or grade (P>0.05). Additionally, we evaluated whether the DNMT3B −579 G>T is associated with CRC patients with alcohol-consuming and smoking status (Table 5). The
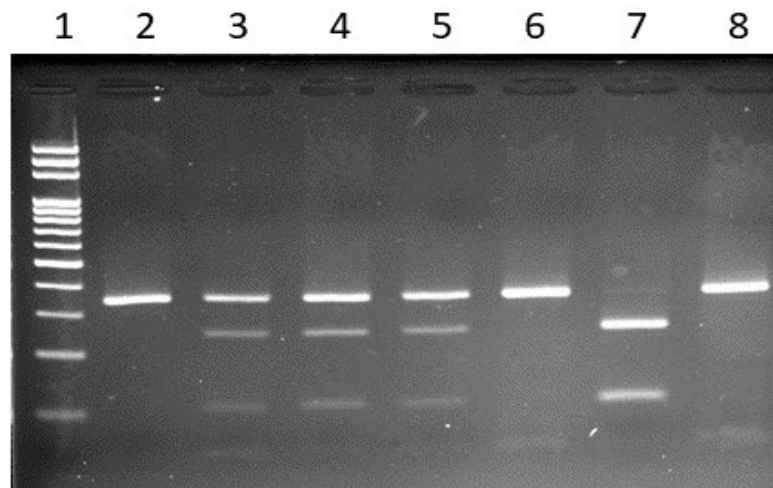

Figure 1. Genotype Distribution of the *DNMT3B* Gene −579 G>T Polymorphism in Agarose Gel. Lane 1: 100 bp DNA Ladder. Lane 2, 6, 8: Wild type GG. Lane 3, 4, 5: Heterozygote GT. Lane 7: Homozygous TT
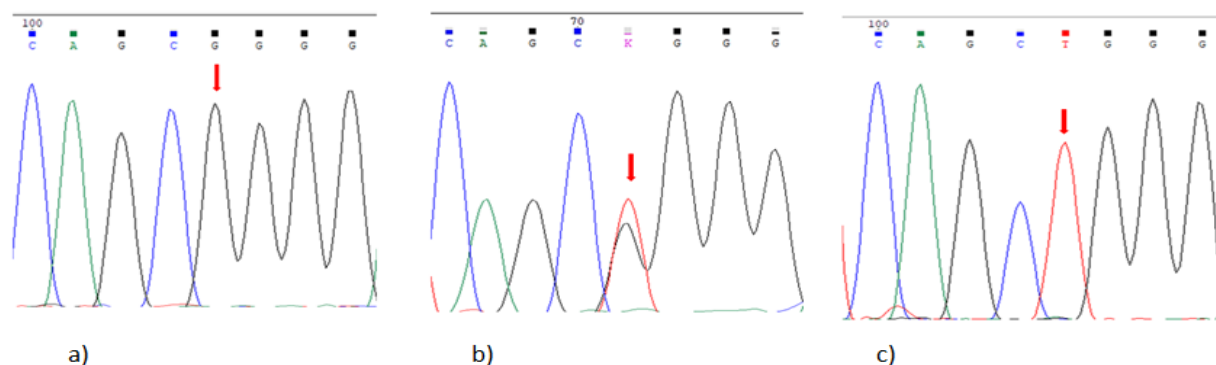
Figure 2. Conformation of Wild-Type GG (a), heterozygous GT (b), and homozygous mutant TT genotype (c) in the sanger sequencing

GT genotype was observed more frequently in smoking (43.8%) and alcohol-consuming (44.4%) patients. However, the mutant TT genotype was detected more frequently in non-smokers and nondrinking patients. Thus, no statistical relationship was determined between smoking and alcohol consumption and the *DNMT3B* gene polymorphism in patients (P>0.05).

Discussion

The DNMT3B is one of the essential DNA methyltransferase enzymes that perform the de novo methylation of DNA (Gagliardi et al., 2018). The promoter −579 G>T polymorphism is located in the exon 1B transcription initiation site of the *DNMT3B* gene, and it has been linked to various diseases, including cancer (Srivastava et al., 2010). In the current study, we evaluated the risk of DNMT3B for CRC and its relationship with clinical parameters, smoking status, and alcohol consumption for the first time in the Azerbaijani population.

We examined the relationship between the −579 G>T polymorphism in the promoter region of the *DNMT3B* gene and colorectal cancer. However, we did not find any significant statistical association between the polymorphism and clinical-pathological parameters, tumor grade and stage, age factor, smoking, and alcohol consumption. In addition, statistical significance was found between GT genotype and reduced CRC risk among male patients in our study.

These findings are supported by similar results revealed by a meta-analysis study involving 3,353 cases and 4,936 controls (Khoram-Abadi et al., 2016). Our results agree with the study conducted on the Chinese population. The researchers noted no statistical association between the clinical-pathological parameters of the disease and the polymorphism. However, the wild-type −579 GG genotype was associated with CRC compared with the mutant TT genotype (Bao et al., 2012). In the study by Perfilyeva et al., it was reported that the GG vs. GT+GG genotype has a role in the development of colorectal cancer in individuals over 60 years of age and in male patients (Perfilyeva et al., 2015). Hong and colleagues identified that the GG+GT genotypes were associated with a lower risk of the disease in male patients

and individuals under 59 years of age, and the researchers suggested that the DNMT3B −579 G>T polymorphism may be a genetic marker in men diagnosed with colorectal cancer (Hong et al., 2007).

Moreover, we determined that the heterozygote GT genotype and the dominant model (GG vs. GT+TT) were associated with a reduced risk of colorectal cancer. Similarly, in a meta-analysis of 18 studies, Xia et al., (2015) reported that the heterozygous GT genotype and the dominant model (GT+TT) were associated with a reduced risk of the disease among the Asian population. A similar conclusion was presented by Zhu et al., (2012) who analyzed 11 studies (including 3513 cases and 3714 controls) to find different results in Asian and European populations. This study highlighted that the DNMT3B promoter −579 G>T polymorphism in Asia was associated with reduced CRC risks; however, no correlation was observed in Europe. Another study supporting our results was presented by Duan et al., (2015) This study was a more comprehensive meta-analysis, including 33 studies and the *DNMT3B* gene variants rs2424913 C/T, rs1569686 G/T, rs6087990 T/C, and rs2424908 T/C were analyzed. According to the results, rs1569686 G/T, rs6087990 T/C, and rs2424908 T/C play a protective role against colorectal cancer in the Asian population. However, the protective role of the DNMT3B −579 G>T has been shown not only in meta-analysis studies but also in case-control studies on patients with colorectal cancer (Fan et al., 2008b).

Our study revealed that the mutant T allele has a protective effect against colorectal cancer. In a study based in China, no statistical correlation was found in head and neck cancer, while a protective association was reported in lung and colorectal cancer (Zhang et al., 2015). Studies in the literature have also shown that the −579 G/T polymorphism of the *DNMT3B* gene is statistically associated with an increased risk of various diseases. It is possible to see these results in early pregnancy loss (Azova et al., 2019), myasthenia gravis (Coppede et al., 2013), and idiopathic thrombocytopenic purpura (Khorshied and El-Ghamrawy, 2012).

On the other hand, studies have indicated that the *DNMT3B* gene polymorphism varies among different races, ethnic groups, and geographic regions. The DNMT3B −579GT genotype is also associated with a

reduced risk of lung cancer (Liu et al., 2012). The results of 13 studies analyzed by Lee and colleagues reported that the rs1569686 type polymorphism of the *DNMT3B* gene plays a protective role against the development of gastric cancer (Li et al., 2016).

In a case-control study of gastric cancer in the Iranian population, Ahmadi et al., (2018) found no association between the DNMT3B −579 G>T polymorphism and the disease. However, they reported an association between tumor grade II and the combined GT/TT genotypes. In addition, no statistical association was reported between the −579 G>T polymorphism of the *DNMT3B* gene and various diseases such as hepatocellular carcinoma (Lao et al., 2013), cervical cancer (Hernandez-Sotelo et al., 2013), esophageal cancer (Fan et al., 2008a), and multiple sclerosis (Yazdanpanahi et al., 2019).

In conclusion, our study shows for the first time that the heterozygous genotype DNMT3B −579 GT and mutant T allele in the Azerbaijani population is associated with a lower risk of CRC. A reduced CRC risk was observed under the dominant model (GG vs. GT+TT), whereas this was not the case with the recessive model (GG+GT vs. TT). The DNMT3B −579 G>T polymorphism was not related to cancer risks at any stage or grade. Our study highlighted that the −579 G>T polymorphism of the *DNMT3B* gene, especially the heterozygous GT variant and mutant T allele, plays a protective role against CRC development.

Author Contribution Statement

None.

Acknowledgements

Conflict of interest
All authors declare that there is no conflict of interest

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