RESEARCH ARTICLE

Methylenetetrahydrofolate Reductase Gene Germ-Line C677T and A1298C SNPs are Associated with Colorectal Cancer Risk in the Turkish Population

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Abstract

Colorectal cancer (CRC) is the third most common cause of death due to cancer in the worldwide and the incidence is also increasing in Turkey. Our present aim was to investigate any association between germ-line methylenetetrahydrofolate reductase (*MTHFR*) C677T and A1298C polymorphisms and CRC risk in Turkey. A total of 86 CRC cases and 212 control individuals of the same ethnicity were included in the current study. Peripheral blood-DNA samples were used for genotyping by StripAssay technique, based on the reverse-hybridization principle and real-time PCR methods. Results were compared in Pearson Chi-square and multiple logistic regression models. The *MTHFR* 677TT (homozygous) genotype was found in 20.9% and the T allele frequency 4.2-fold increased in CRC when compared with the control group.The second SNP *MTHFR* 1298CC (homozygous) genotype was found in 14.0% and the C allele frequency 1.4-fold elevated in the CRC group. The current data suggest strong associations between both SNPs of germ-line *MTHFR* 677 C>T and 1298 A>C genotypes and CRC susceptibility in the Turkish population. Now the results need to be confirmed with a larger sample size.

Keywords: *MTHFR* gene - SNPs - C677T and A1298C - colorectal cancer risk - Turkey

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Introduction

Colorectal cancer is one of the most common types of cancer and the incidence varies and appears to be increasing in worldwide. Recent literature findings showed that the ethiological parameters such as; chromosomal rearrengements, point mutations, gene polymorphisms, environmental factors, lifestyles, and epigenetic alterations may cause to the CRCP (Fearon et al., 1990; de Kok et al., 2000; Parkin et al., 2002; Ulrich et al., 2002). The folate status in some cases was also reported in the CRC risk (Choi et al., 2000; Xu et al., 2004). The methylene tetrahydrofolate reductase (*MTHFR*) enzyme plays a crucial role in the folate metabolism that catalyzes the irreversible reaction of 5,10-methylene-tetrahydrofolate to 5-methyl tetrahydrofolate, which serves as a substrate for the remethylation of homocysteine to methionine, with the subsequent synthesis of S adenosylmethionine(Friedman et al., 1999; Parle et al., 2006; Slattery et al., 1999; Pardini et al., 2011). The substrate of *MTHFR* 5,10-methylene-tetrahydrofolate, is also required for thymidine synthesis via thymidylate synthase, and indirectly for purine biosynthesis(Frosst et al., 1995; Weisberg et al., 1998). Decreasing the *MTHFR* enzyme function may cause to the global DNA hypomethylation due to lack of the intracelular methyl sources and initiates carcinogenesis process. (Blount et al., 1997; Stern et al., 2000; Fang et al., 2003).

Two common polymorphic SNPs were reported in exons 4 and 7 of *MTHFR* gene; one is defined in codon 677 C<T and the other one is in codon 1298 A<C.The first C677T SNP, positioned in exon 4 leading to an alanine to valine conversion Goyette et al., 1998). Individuals with homozygous *MTHFR* TT genotype have 30% and heterozygous carriers show 65% enzyme activity (Frosst et al., 1995). The TT genotype is also associated with higher plasma homocysteine and reduced plasma folate levels (Delougheryt et al., 1996; Schwartz et al., 1997). The second SNP in *MTHFR* gene, A1298C (rs1801131) in exon 7, lead to a glutamate to alanine substitution at codon 429 (E429A), (van der Put et al., 1998; Chen et al., 2002; Weisberg et al., 2002). This polymorphism lies in the C-terminal end of the enzyme, the S-adenosylmethionine regulatory domain, and may result in a decrease of 40% in enzyme activity of the variant genotype. Nevertheless, this variant has not associated either with a thermolabile enzyme or with alterations in the levels of homocysteine in the plasma (Weisberg et al., 2002). Both mutated *MTHFR*

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gene profiles were reported in some cancer types (Izmirli et al., 2011; Izmirli et al., 2013). There are conflicting results that rangin from strong linkage and no association about *MTHFR* and CRC risk in different literature findings.

In the current case-control study, it was aimed to describe the effect of polymorphic germ-line folate-associated gene *MTHFR* markers (C677T and A1298C) in colorectal carcinoma in Turkish population.

Materials and Methods

Patients, clinical diagnosis and laboratory assessment

In a total of 86 CRC patients; 70 colonal (81.4%) and 16 rectal (18.6%), 54 male (62.8%), 32 female (37.2%) and the mean age-min-max; 63.22±30.41 (45-87) were included in the current report.The tumoural tissue samples were used for tissue specific proto-oncogene *KRAS* genotyping and epigenetic profiling (data not shown) and peripheral blood-EDTA samples from each patients were used for germ-line *MTHFR* gene profiling.The results belong to the germ-line mutation profiles for *MTHFR* gene were compared to the healthy individuals from the same ethnicity (Ozdemir et al., 2012). Peripheral blood-DNA samples were obtained during routine diagnosis from CRC patients in Cumhuriyet University Training and Research Hospital by the collaboration of department of medical genetics and general surgery between June 2007 and January 2010.Samples were used for genotyping for point mutations of C677T and A1298C markers for *MTHFR* gene. Informed consent was obtained from all of the patients and control group individuals.

Mutation analysis

Peripheric blood tissues containing EDTA from patients and control group were used for genomic DNA isolation.The total genomic DNA was extracted by the MagnaPure Compact (Roche) and Invitek kit extraction techniques (Invitek®; Invisorb spin blood, Berlin, Germany). Target genes were simultaneously amplified in a biotin-labelled single multiplex amplification reaction (Viennalab®; PGX-HIV StripAssay, Vienna, Austria) which is based on the reverse-hybridization principle automatically and by Real Time PCR,LightCycler 2.0 methods (Roche). The multiple polymerase chain reaction (PCR) was performed in a Perkin Elmer 9600 and the profile consisted of an initial melting step of 2 min at 94°C; followed by 35 cycles of 30s at 94°C, 30s at 61°C, and 30s at 72°C; and a final elongation step of 7min at 72°C for stripAssay genotyping. High portion of samples were also analysed by real-time PCR technique (LightCycler 2.0, Roche). Briefly, LightCycler FastStart DNA Master HybProbes, master mix and DNA template were used for real-time amplification.The amplification conditions for 45 cycles were; denaturation in 95°C for 10 seconds, annealing for 5-20 seconds, extension in 72°C, melting curve step with denaturation in 95°C, annealing for 30 seconds, melting in 95°C and cooling step in 40°C for 30 seconds.Software programme (LightCycler 2.0, Roche) was used for detection of the mutated and normal genotype profiles of target genes in the current CRC and healthy controls.

Statistical analysis

Alternative genotype frequencies for mutated *MTHFR* markers in patients of CRC and controls were compared using pearson Chi-square and multiple logistic regression analysis. Statistical analysis was performed using SPSS version 16 (SPSS, Chicago, IL, USA). A value of $P<0.05$ was considered as statistically significant and mutated T allele frequency was discussed in the current report.

Results

Presented results show germ-line variations in *MTHFR* gene. Peripheral blood-EDTA samples from healthy controls and CRC patients were examined for genotyping in the current study.In a total of 86 CRC patients [(54 male (62.8%) and 32 female (37.2%)] of 70 (81.4%) colonal, 16 (18.6%) rectal cancer mean age 63.22±30.41 (45-87) were compared to the 212 healthy individuals from the same population (Ozdemir et al., 2012). The number of patients according to tumour type were; 72 (83.7%) adenocarcinoma, 11 (12.8%) mucinous type carcinoma, 2 (2.3%) tubulovillous adenoma and 1 (1.2%) glandular type tumour (Table 1). The investigated patients according to differentiated tumour types were; 38 (44.2%) non-differentiated, 6 (7.0%) poor type differentiated, 31 (36.0%) intermediate type and 11 (12.8%) advance tumour type (Table 1). Most of tumour were in grade 2 in the current CRC cohort histopathologically; 9 (10.5) samples were in grade1, 54 (62.8%) samples were in grade 2, 8 (9.3%) samples were in grade 3 and 7(8.1%) samples were in unknown profile (Table 1). Clinical inspection and histopathologic evaluation revealed that 53 (61.6%) patients had lymph node metastasis, 2 (2.3%) patients had bone and the rest of the patients (28/32.6%) had no

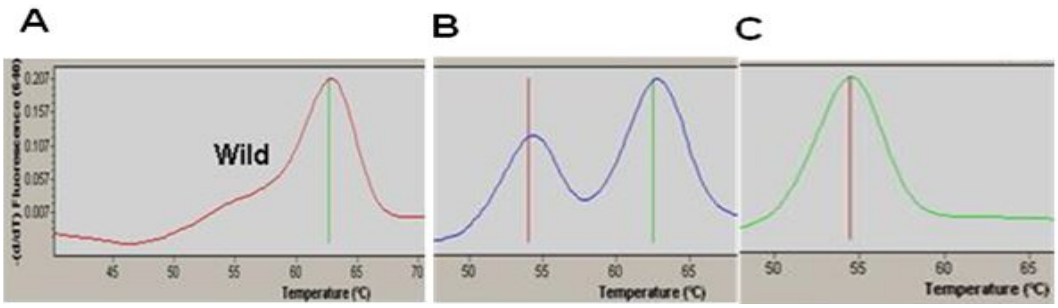

Figure 1. Real Time Melting Profiles of Wild A), Heterozygous B) and Homozygous Mutated C) *MTHFR* 677A>C SNP in Current CRC Tumours

metastasis (Table 1).

The germ-line T allele frequency of 677 C>T and 1298 A>C SNPs for *MTHFR* gene has been shown to be a risk factors for CRC in the current cohort from Turkish population (Figure 1), (Table 1). The distribution of genotype frequencies of the studied polymorphisms between the patients with CRC and control groups was in Hardy-Weinberg equilibrium. The prevalence of genotypes for *MTHFR* gene C677T SNP in patients with CRC (41.9% for CC, 37.2% for CT and 20.9% for TT respectively) was higher than the control group (73.0% for CC, 27.0% for CT and 0.0% for TT respectively). The prevalence of genotypes for *MTHFR* gene for the second SNP of 1298C in patients with CRC (31.4% for AA, 54.6% for AC and 14.0% for CC respectively) was also higher than the control group (34.0% for AA, 66.0% for AC and 0.0% for CC respectively), (Table 2). The T allele frequency of codon 677 SNP was 0.395 for CRC patients and 0.130 for healthy individuals in the current results. *MTHFR* 677TT (homozygous) genotype was found 20.9% and T allele frequency 4.2-fold increased in CRC when compared to the control group form the same ethnicity. (OR: 4.2, CI: 2.78-6.36), p<0.0001.

Similar findings were detected in the second SNP marker (A1298C) of *MTHFR* gene in the current CRC cohort. The C allele frequency of codon 1298 SNP was 0.413 for CRC patients and 0.340 for healthy individuals in the current results. *MTHFR* 1298CC (homozygous) genotype was found 14% and C allele frequency 1.42-fold increased in CRC when compared to the control group form the same ethnicity (OR: 1.42, CI: 0.99-2.05), p<0.0001.That both differences were statistically significant when compared to the control group (Table 2).

Table 1. Some Clinical Characteristics Such as; the Mean age, Gender, Cancer Types of current CRC Patients from Turkish Population

Clinical Characteristics		CRC Patients (n:86) No.	%
The mean age		63.22±30.41 (45-87)	
Gender	M	54	62.8
	F	32	37.2
Material type	Pheripheral blood - EDTA,	86	100
Cancer type	Rectal	16	18.6
	Colonal	70	81.4
Tumour type	Adenocarcinoma	72	83.7
	Mucinous	11	12.8
	Tubulovillous adenoma	2	2.3
	Glandular	1	1.2
Differentiation type	Advance	11	12.8
	Intermediate	31	36.0
	Poor	6	7.0
	Non-differentiated	38	44.2
Grade	Grade 1	9	10.5
	Grade 2	54	62.8
	Grade 3	8	9.3
	High Grade	8	9.3
	Unknown	7	8.1
Metastasis	Non-metastasis	28	32.6
	Lymph node	53	61.6
	Bone	2	2.3
	Other	3	3.5

Table 2. The Prevalence of Genotypes and T Allele Frequency of *MTHFR* 677 C >T and C Allele Frequency of *MTHFR* 1298 A>C SNPs in the Current CRC Cohort and Healthy Controls

Gene/Genotype		Patients (n:86) No.	%	Controls* (n:212) No.	%
MTHFR C677T	C/C	36	41.9	207	97.6
	C/T	32	37.2	5	2.4
	T/T	18	20.9	0	0.0
Alleles	C	104	0.605	419	0.988
	T[a]	68	0.395**	5	0.012
MTHFR A1298C	A/A	27	31.4	209	98.6
	A/C	47	54.6	3	1.4
	C/C	12	14.0	0	0.0
Alleles	A	101	0.587	421	0.993
	C[b]	71	0.413***	3	0.007

*Ozdemir et al., 2012; **The T allele was significant for *MTHFR* C677T; Odds Ratio=4.2 (2.78-6.36), p<0.0001; ***The C allele was also significant for *MTHFR* A1298C; Odds Ratio=1.42 (0.99-2.05)), p<0.0001; [a]p value <0.0001: Odds ratio=4.2: CI (95%) (2.78-6.36); [b]p value <0.0001: Odds ratio=1.42: CI (95%) (0.99-2.05)

Discussion

Most of the CRC cases are sporadic but approximately 25% of patients have a familial history. Multiple ethiological parameters such as; genetic variation, epigenetics, socioeconomic, demographic, racial and ethnic diversity impacts on outcomes of disease and must be carefully considered in CRC evaluation (Kono et al., 2005; Cheah et al., 2009). However, the exact molecular trigger mechanisms of CRC has not been completely defined yet. The systemic functional gene *MTHFR* is a one of the trigger molecule in human complex diseases including CRC (Ge et al., 2012). Gene has pluripotent affect on folate metabolism, cells methyl sources, gene regulation, DNA methylation, maintain the integrity and stability of DNA (Ge et al., 2012). Literature findings show conflicting results about *MTHFR* role in oncogenesis. In several studies, the 677TT genotype was associated with an increased risk of CRC (Gallegos - Arreola et al., 2009; Sameer et al., 2011). Guerreiro et al. (2008) have found that the TT genotype is associated with an increased risk for CRC in Portuguese population but no relationship was reported in district region of Turkish population (Zeybek et al., 2007). The results about *MTHFR* A1298C SNP on CRC risk are also conflict (Chang et al., 2007; Zeybek et al., 2007; Cao et al., 2008). For the 1298 CC genotype, two studies reported an increase in risk (Kury et al., 2008; Lightfoot et al., 2008), while other some meta-analyses suggest that 1298 CC genotypes may be associated with a decrease in CRC risk (Huang et al., 2007; Fernandez-Peralta et al., 2010; Li et al. 2011) in Chinese Han population. Nassiri et al (2013) have claimed that the SNPs in *MTHFR* gene that associated with colorectal cancer can be used as a potential genetic marker tool for improving cancer diagnosis and treatment. Yousef et al (2013) have also claimed that the 677 SNP and the TA haplotype of *MTHFR* gene may modulate the risk for CRC development among the Jordanian population. But, lower risk were reported for *MTHFR* 677T allele in Asian (Yang et al., 2013) and Spanish populations (Huang et al., 2007).

In the current study similar findings were detected in the second SNP marker (A1298C) of *MTHFR* gene in the current CRC cohort. The germ-line T allele for codon 677 C>T and C allele frequencies of codon 1298 A>C SNPs has been shown to be a risk factors for CRC in the

current cohort from Turkish population. The prevalence of genotypes for *MTHFR* gene C677T SNP in patients with CRC (41.9% for CC, 37.2% for CT and 20.9% for TT respectively) was higher than the control group The T allele frequency of codon 677 SNP was 0.395 for CRC patients and 0.130 for healthy individuals in the current results. *MTHFR* 677TT (homozygous) genotype was found 20.9% and T allele frequency 4.2-fold increased in CRC when compared to the control group form the same ethnicity. (OR: 4.2, CI: 2.78-6.36), p<0.0001.

The C allele frequency of codon 1298 SNP was 0.413 for CRC patients and 0.340 for healthy individuals in the current results. *MTHFR* 1298CC (homozygous) genotype was found 14% and C allele frequency 1.42-fold increased in CRC when compared to the control group form the same ethnicity (OR: 1.42, CI: 0.99-2.05), p<0.0001.That both differences were statistically significant when compared to the control group (Table 2). Some of the previous studies demonstrate that notable difference between patients and controls whereas remaining studies found that there was no association for both of polymorphisms. The different results regarding the effects of those genetic polymorphisms on CRC risk can be ascribed to the differences in racial origin of the population, the lifestyle, and the pattern of diet in distinct countries (Zhao et al., 2014). Recent literature findings reflect an importance of genes involved in folate metabolism in complicated diseases and cancer risk by pleiotropic effect (Yousef et al., 2013; Ozdemir et al., 2014). Torre at al. (2014) have claimed that polymorphic 677TT *MTHFR* SNP with other conditions such as; smoke, high HbA1c levels and dyslipidaemia are increased colorectal tumour risk. Here we report increased germ-line mutation profiles in both polymorphic *MTHFR* gene SNPs in CRC patients in Turkish population.

It is well known that, many parameters such as; oncogenes, epigenetic alterations in tumour suppressor genes, viruses and many other intrinsic and/or extrinsic environmental factors may directly play crucial role in CRC progression.The current results with some of previous literature findings have pointed out the importance of the functional gene mediated CRC progression. Presented results show germ-line variations in *MTHFR* gene that the homozygous individuals for *MTHFR* 677TT and 1298CC SNPs are more likely to develop CRC than those with wild-type genotype.

In conclusion, current results indicates susceptibility of germ-line C677T and A1298C SNPs in systemic functional gene of *MTHFR* that associated with CRC risk in Turkish population. It is also possible to assumed that parents with mutated for both SNPs may contribute CRC risk in their offsprings by germ-lime mutated allele inheritance.

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