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The Methionine Synthase Reductase (MTRR) A66G and Methionine Synthase (MTR) A2756G Polymorphisms Might be a Risk Factor for Colorectal Cancer in People Living near the Southern Coast of the Caspian Sea

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ABSTRACT

A case-control study was designed to investigate the association between the risk of colorectal cancer and genetic variation in four single-nucleotide polymorphism (SNPs) within involved genes in folate metabolism: C677T and A1298C of methylenetetrahydrofolate reductase (MTHFR), A66G of methionine synthase reductase (MTRR), and A2756G of methionine synthase (MTR). Genomic DNA was extracted from peripheral blood of 50 patients with newly diagnosed colorectal cancer and 100 non-cancer controls. The statistical analysis was carried out by logistic regression. The results showed that the MTR and MTRR SNPs were significantly associated with increased risk of colorectal cancer ($p<0.01$). Moreover, no significant relation was found between MTHFR C677T and MTHFR A1298C polymorphisms and the risk of colorectal cancer. These findings suggest that the MTRR A66G and MTR A2756G polymorphisms might be some genetic risks factor for colorectal cancer in the studied population.

Keywords: Colorectal Cancer; Gene Polymorphism; Methylenetetrahydrofolate Reductase; Methionine Synthase Reductase; Methionine Synthase

1. Introduction

Folate has shown a key role in the etiology of colorectal cancer [1]. The leading cause of colorectal cancer might be the genetic differences in the folate metabolic pathways, such as single nucleotide polymorphisms (SNPs) [2]. Folate converts into one of the three forms within the cells, namely 5-methyl-THF (Tetrahydrofolate), 5,10 methylene-THF, or 10-formyl-THF. In this regard, 5-methyl-THF is the dominant circulating form of folate, which is used for processes such as DNA methylation reactions. Also, 5-methyl-THF donates a methyl group to homocysteine which is converted it to methionine. Methionine is later catabolized to produce S-adenosylmethionine (SAM), a major source of methyl groups used for DNA methylation. The second folate form (i.e., 5,10 methylene-THF) changes deoxyuridylate (dUMP) into thymidylate (dTMP) and the last folate form (i.e., 10-formyl-THF) produces new purines. Purines are used both in RNA and DNA synthesis. Furthermore, dTMP is produced for DNA synthesis and DNA repair or DNA replication [3] (Figure 1). The enzyme that converts 5,10 methylene-THF to 5-methyl-THF is MTHFR (Figure 1). The MTHFR gene includes two polymorphisms: C677T and A1298C. The MTHFR variant C677T causes a decrement in enzyme activity, leading to differential methylation of genomic DNA [4,5], which is associated with different forms of cancer. Also, the incremental change in the plasma level of homocysteine could be caused by reduced MTHFR activity. Homocysteine may also affect methylation of DNA [6]. The second MTHFR variant (i.e., A1298C) also causes conformational modifications within the MTHFR enzyme that alter the enzymatic activity [7,8]. The remethylation of homocysteine to methionine which is needed for SAM production is catalyzed by MTR (A66G). In methylation process, vitamin B_{12} is used as a cofactor (A66G) by MTR. MTRR (A2756G) may catalyze the reductive methylation of vitamin B_{12} using SAM as a methyl donor and leads to the activation of MTR [9,10].

The deficiency of folate metabolism due to dietary, pharmacological, and genetic factors has been suggested to increase the risk of colorectal cancer via aberrations in DNA methylation and imbalance of DNA precursors [11,12]. In this respect, some environmental factors such as geographical and ethnic backgrounds are extremely important to capture biodiversity and fully understanding risk and treatments related to polymorphism [13]. In the present study, we proposed to examined the correlations between polymorphism of C677T and A1298C within methylenetetrahydrofolate reductase (MTHFR), A66G within methionine synthase reductase (MTRR), and A2756G within methionine synthase (MTR) and colorectal cancer occurrence in people living in the South of Caspian Sea (Mazandaran pr ovince, Iran).

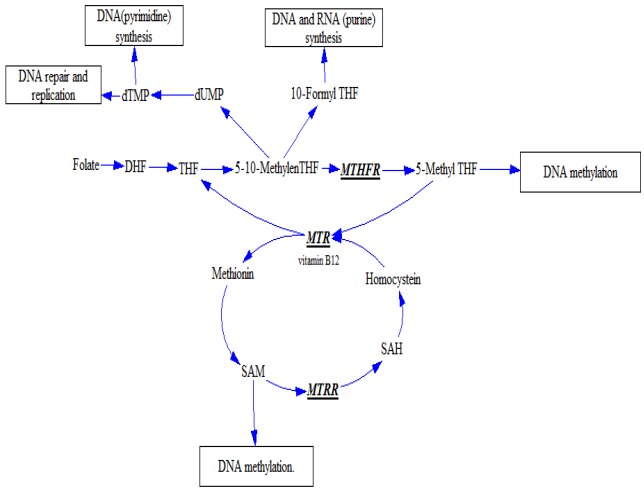

Figure 1. Folate cycle and the role of methylenetetrahydrofolate reductase (MTHFR), methionine synthase reductase (MTRR), and methionine synthase (MTR) gene in the metabolism of folate (DHF, dihydro tetra folic acid; THF, tetrahydrofolic acid; 5-10-MethylenTHF,5-10-methylenetetrahydrofolate;5-Methyl THF,5-methyltetrahydrofolate; SAM, S- adenosylmethionine).

2. Materials and Methods

2.1. Study population and sample collection

50 patients with nonhereditary colorectal cancer diagnosed after a colonoscopy at Imam Hospital (Sari, Iran) between April 2019 and August 2020 were study cases of this experiment. The controls included 100 blood donors with the same age and blood collection year Each patient gave us their Informed consent. The study was approved by the Medical Ethics Committee of Sari Medical Sciences Branch, Islamic Azad University. The mean age of the patients was 55±18 years for women and 59±15 years for men. People with a history of various risk factors for chronic disease

and other types of genetic and familial malignancies were excluded. All samples were collected randomly. Blood samples were collected in EDTA tubes and latedr were immediately centrifuged and separated for plasma and stored at -20℃. The DNA was extracted from whole blood samples by DNG plus Genomic DNA extraction kit (Cinnagen, Iran) according to the manufacturer's instructions..

2.2.Genotyping of the MTHFR, MTR, and MTRR genes

Genotyping for four SNPs (MTHFR C677T, MTHFR A1298C, MTR A2756G, and MTRR A66G) was carried out using polymerase chain reaction (PCR) in order to amplify blood DNA followed by restriction enzyme digestion and gel electrophoresis. DNA amplification of C677T fragment by PCR was performed using two primers: The forward primer and the reverse primer. The fragment was amplified in 25 µl PCR mixture containing 1 µM of template DNA, 0.2 µl *Tag* DNA polymerase, 0.5 µl dNTPs mix, 1 µM forward primer, 1 µM reverse primer, and 1.5µM $MgCl_2$. Polymorphisms were identified by the enzymatic digestion of the obtained fragment by PCR amplification with a restriction endonuclease. The polymorphisms create a restriction site for the enzymes. The enzymatic digestion, which was acquired in a 10-µl reaction mixture, consisted of the following materials: 1X NE buffer (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.9@ 25℃) and 5 units of HinfI/ 10µl reaction mixture. The mixture was incubated at 37℃ for enzymatic digestion for 3 h. The products of enzymatic digestion were separated by electrophoresis in 3% agarose gel stained with ethidium bromide. The sequences of the primers, annealing temperatures, restriction enzymes, genotype, and length of the restriction fragments (bp) are listed in Table 2.

Table1. Background Characteristics of Patients by Age and Sex

Characteristic	n (%)
Total patients	
Males	**28(56)**
Females	**22(44)**
Age(in years)	
Mean	**57±16.2**
Median	**57**
Range	**37-74**

Table 2. The Sequences of the Primers, Annealing Temperatures (Tm), Restriction Enzymes, Genotype, and Length of the Restriction Fragments (bp)

SNPs	Primer sequences	Tm	Restriction enzymes	Genotype and Fragment size (bp)		
				Normal Homozygous	Heterozygous	Homozygous
MTHFR C677T	F:(5′-TGAAGGAGAAGGTGTCTGCGGGA-3′) R:(5′-AGGACGGTGCGGTGAGAGTG-3′)	59°C	*Hinf 1*	CC(86 bp)	CT(86,50,36)	TT(50, 36)
MTHFR A1298C	F:(5′-GAGCTGAAGGACTACTACCTC-3′) R:(5′-CACTTTGTGACCATTCCGGTTTG-3′)	61°C	*MboII*	AA(25,28,31)	AC(59,31,28,25)	CC(59,25)
MTR A2756G	F:(5′-GAACTAGAAGACAGAAATTCTCTA-3′) R:(5′-CATGGAAGAATATCAAGATATTAGA-3′)	61°C	*HaeIII*	AA(66)	AG(66,37,29)	GG(37,29)
MTRR A66G	F:(5′-CAGGCAAAGGCCATCGCAGAAGACAT-3′) R:(5′-CACTTCCAACCAAAATTCTTCAAAG-3′)	59°C	*NdeI*	AA(44,22)	AG(66,44,22)	GG(66)

Tm: Annealing temperature; F: Forward; R: Reverse; bp: base pairs

2.3. Statistical analysis

Statistical analyses and computations were done using the R software [14]. Allele and genotype frequencies and Hardy-Weinberg equilibrium for the SNPs were estimated using the GENETICS package [15] in R. Associations between SNPs genotypes and colorectal cancer were performed using a multivariable logistic regression model including all four SNPs genotypes, sex and age variables to adjust their confounding effects. Odds ratios and their 95% confidence interval were calculated based on the output of logistic regression analysis. All p-values less than 0.05 were

considered as statistically significant.

2.4. Ethical statement

This study was approved by the Institution Ethics Committee approval with the number of IR.IAU.SARI.REC.1398.079 and all participants provided informed consent. The online version of the approved version is available to the public at the address http://ethics.research.ac.ir/IR.IAU.SARI.REC.1398.079 .

3. Results

3.1. Distribution of genotypes in patients and controls

The general characteristics of the studied patients are presented in Table 1. The frequencies of alleles and genotypes of the MTHFR C677T, MTHFR A1298C, MTR A2756, and MTRR A66G loci are shown in Table 3. The chi-square ($\chi 2$) test demonstrated that the distributions of MTRR and MTHFR A1298C loci genotypes deviated from Hardy-Weinberg equilibrium ($p<0.05$). Association parameters (odds ratios (OR), 95% confidence intervals (CI), and p-values) adjusted for the effects of age and sex are given in Table 3. We examined the relation between the four common polymorphisms of three critical enzymes involved in folate metabolism and the risk of colorectal cancer. Our result suggested that no significant relation was found between MTHFR C677T and MTHFR A1298C polymorphism and the risk of colorectal cancer. The presence of C677T mutation (CT-genotype, TT-genotype and T-allele) was not a significant risk factor for colorectal cancer. Results for patients with the A1298C mutation (AC-genotype, CC-genotype and C-allele) is similar to the C677T mutation. Unlike MTHFR C677T and MTHFR A1298C, the genotype and allele frequencies for MTR A2756G and MTRR A66G differed significantly between patient and control groups. In MTR A2756G polymorphism, GG genotype had the highest risk (OR = 7.41, 95%CI = 1.96-27.9, p = 0.003) for the colorectal cancer. Genotypes carrying the G allele (AG + GG) also showed significant risk (OR = 3.18, 95%CI = 1.34-7.52, p = 0.008). The G allele was significantly associated with colorectal cancer (OR =2.55, 95%CI = 1.36-4.80, p = 0.003). Moreover, in MTRR A66G polymorphism, the GG genotype was associated with an increased risk of colorectal cancer (OR = 107.6, 95%CI = 12.45-929.9, p < 0.0001). The carriers of G (AG + GG) were at a high risk (OR = 9.34, 95%CI = 4.06-21.50, p < 0.0001). Finally, a strong association was found between allele G and colorectal cancer (OR =6.63, 95%CI = 3.40-12.94, p < 0.0001).

Table 3. Genotype Frequencies in Case and Control Groups, Odds Ratios (OR) and 95% CIs for MTRR, MTR, and MTHFR Polymorphisms Adjusted for the Effects of Sex and Age

Genotype/Allele	Control n=100 (%)	Case n=50 (%)	OR	95%CI	p value
MTHFR C677T					0.39
CC	69 (69)	32 (64)	1	Reference	-
CT	30 (30)	16 (32)	1.11	0.40-3.07	0.84
TT	1 (1)	2 (4)	3.49	0.17-70.6	0.41
CT + TT	31(31)	18(36)	1.09	0.44-2.71	0.85
C	168(84)	80(80)	1	Reference	-
T	32(16)	20(20)	1.22	0.49-3.04	0.66
MTHFR A1298C					0.57
AA	81 (81)	34 (68)	1	Reference	-
AC	16 (16)	12 (24)	1.65	0.51-5.30	0.40
CC	3 (3)	4 (8)	2.31	0.32-16.4	0.40
AC + CC	19(19)	16(32)	1.97	0.74-5.26	0.17
A	178(89)	80(8)	1	Reference	-
C	22(11)	20(2)	1.54	0.68-3.49	0.29
MTR A2756G					0.007
AA	60 (60)	17 (34)	1	Reference	-
AG	34 (34)	21 (42)	2.72	1.01-7.40	0.049
GG	6 (6)	12 (24)	7.41	1.96-27.9	0.003
AG + GG	40(40)	44(66)	3.18	1.34-7.52	0.008
A	154(77)	55(55)	1	Reference	-
G	46(23)	45(45)	2.55	1.36-4.80	0.003
MTRR A66G					<0.0001
AA	83 (83)	17 (34)	1	Reference	-
AG	16 (16)	16 (32)	3.78	1.44-9.88	0.007
GG	1 (1)	17 (34)	107.6	12.45-929.9	<0.0001
AG + GG	17(17)	33(66)	9.34	4.06-21.50	<0.0001
A	182(91)	50(50)	1	Reference	-
G	18(9)	50(50)	6.63	3.40-12.94	<0.0001

4. Discussion

Colorectal cancer rates third among the most common cancers with 1.8 million new cases and the fourth rate in the most common causes of

cancer death in 2018 in the world [16]. According to data from the National Cancer Registry of Iran and the Center for Disease Control and Prevention of the Ministry of Health and Medical Education of Iran, the central, northern, and western provinces of Iran have the highest incidence rates of colorectal cancer [17]. However, it is the most often diagnosed cancer and the main cause of cancer death. The mortality rate of the disease varies across the countries and depends on the degree of economic development, social and lifestyle factors [16]. In addition to environmental factors, genetic factors such as genetic polymorphisms of enzymes involved in folate metabolism might be the risk factors for this cancer. Among these enzymes, MTHFR, MTR, and MTRR are risky genes.

In the present study, the associations of MTHFR C677T and A1298C, MTRR A66G, and MTR A2756G polymorphisms with the susceptibility of colorectal cancer were analyzed. The results showed that the MTR 2756AG genotype, 2756GG genotype, 2756 AG and GG genotype, and 2756 G-allele were significantly associated with increased risk of colorectal cancer and MTRR 66AG genotype, 66GG genotype, 66 AG and GG genotype, and 66 G-allele are strong risk factors for colorectal cancer. No significant association was found between MTHFR C677T and MTHFR A1298C polymorphisms and the risk of colorectal cancer.

There are conflicting results from previous colorectal cancer studies about the relationship between the polymorphisms of the three investigated genes and the risk of colorectal cancer. Several studies have shown the associations between genotypes of MTHFR, MTRR, and MTR with increased risk of colorectal cancer [18-22] but in other studies no relationship was found [23]. On the contrary, some studies have reported even reverse associations with reduced risk of colorectal cancer and even protective effect on it [24-30] depending on adequate dietary folate intake. Furthermore, some studies show low plasma folate levels were associated with a lower risk of colorectal cancer [31]. They concluded that folic acid supplementation (1 mg/d) did not prevent the recurrence of colorectal adenomas and tended to increase greater adenoma multiplicity. Nevertheless, in another trial, folic acid supplementation was associated with lower recurrence of adenomas among those with low plasma folate at baseline [32]. in a study of nutrients, foods, and colorectal cancer results manifested thatfolate intake lasted through diet or supplements had a complex relationship with colorectal cancer risk; it potentially promoted the growth of pre-existing tumors while inhibited the formation of new tumors in healthy tissue [33]. Mokarram *et al.* showed that the MTHFR 677TT genotype combined with a high serum folate statusmight be a risk factor for tumor-specific gene promoter hypermethylation among colorectal patients of southern Iran[34].

in another study by Naghibalhossaini *et al.* on the same people, MTHFR 667T allele was strongly correlated with colorectal cancer and microsatellite instability. They proposed that it might be mediated by the inactivation of mismatch repair genes caused by their promoter hypermethylation They also found no notable difference in the MTHFR 1298C allele. They concluded that it could be due to no significant alteration in the activity of the enzyme caused by this polymorphism [35]..

Similar to multifactor disorders, colorectal cancer is affected by both genetic and environmental factors and the effect of genotype may differ in populations with different amounts of folate intake [36]. So, many factors, like dietary folate supply, alcohol intake, age, and gender may modify the effects of these gene polymorphisms and the risk of colorectal cancer [37].

polymorphisms measurement in four SNPs of three genes simultaneously was the aim of in this case-control study, however, the present study suffered from some limitations; e.g., the lack of measurement of plasma folate and folate levels in red blood cells and folate in the diet. Another limitation of the present research was the few number of examined samples due to executive constraints. The few examining population could reduce the power of the statistical analysis. Therefore, to avoid further barrier, we did not consider further analyses in each subgroup. Since this study is not generalized, the results should be explicated cautiously. Hence, it is recommended to perform similar studies with an extended sample size and further evaluation in terms of

micronutrient status for various ethnic groups.

Decleration of interest: none

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