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ORIGINAL ARTICLE

Amino acid substitution polymorphisms of two DNA methyltransferases and susceptibility to sporadic colorectal cancer

Fatemeh Khatami [1], Seyed Reza Mohebi [1], Somayeh Ghiasi [1], Mahdi Montazer Haghighi[1], Azadeh Safaee [1], Mohammad Hashemi [2], Mohammad Reza Zali [1]

[1] *Research Institute for Gastroenterology and Liver Disease, Shahid Beheshti University, M.C., Tehran, Iran*

[2] *Department of pathology, Imam Hossein Hospital, Shahid Beheshti University, M.C., Tehran, Iran*

ABSTRACT

Aim: To evaluate the association of DNMT1 and MGMT amino acid substitution polymorphisms and colorectal cancer in Iranian population.

Background: The MGMT and DNMT1 are two important methyltransferase enzymes. Amino acid substitution polymorphisms in MGMT and DNMT1 genes may be associated with the genetic susceptibility to sporadic colorectal cancer.

Patients and methods: We assessed eight non-synonymous polymorphisms of these two genes by PCR/Pyrosequencing. Our population consisted of 208 individuals with sporadic colorectal cancer and 213 controls. Allele frequencies and genotypes were compared between the cases and controls.

Results: Odds ratios were calculated and there was no association between DNMT1 and colorectal cancer. However, there was a significant association between two polymorphisms with sporadic colorectal cancer; Arg128Gln (OR 5.53, 95%CI 2.58-7.16) and Gly160Arg (OR 3.04, 95%CI=1.48-6.31) of MGMT gene.

Conclusion: This finding could be an accurate indicator of high occurrence of colorectal cancer in Iranian patients.

Keywords: *O6-methylguanine-DNA methyltransferase (MGMT), DNA methyl transferase 1 (DNMT1), amino acid substitution polymorphisms, pyrosequencing.*

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INTRODUCTION

Colorectal carcinoma (CRC) is the second leading cause of cancer related deaths in the Western world. Colorectal cancer is the third most common cancer and 30% of people with CRC will die of it (1).

Genomic stability is maintained by DNA repair genes in dividing cells. An individual's capacity to repair DNA is genetically determined and is the result of combinations of multiple genes with different activities. Single nucleotide polymorphisms (SNPs) are common allelic variants occurring approximately once per every 500 to 1000 base pairs in the human genome (2). Single nucleotide polymorphisms (SNPs) can result in small structural alterations in some important enzymes and therefore changes in the susceptibility to cancer. Several polymorphic genes have been associated with modification of

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Reprint or Correspondence: Fatemeh Khatami, Research Institue for Gastroenterology and Liver Diseases, Shahid Beheshti University, M.C., Iran
E-mail: Khatam81@yahoo.com

susceptibility to diseases such as cancer (3,4). One procedure of DNA repair is methylation of the O6-atom of guanine base residues as a major pre-mutagenic lesion in DNA produced by endogenous as well as exogenous alkylating agents. The persistence of O6-methylguanine during DNA replication may cause a G:C to A:T transition (5), because O6-methylguanine residues preferentially pair with thymine during DNA synthesis. The O6-methylguanine DNA methyltransferase (MGMT; MIM# 156569; GDB: 125264) gene is responsible for repairing the alkylation DNA damage, and removes alkylating groups at the position O6 of guanine; therefore, it plays an important role in maintaining the normal cell physiology and genomic stability.

Loss of expression of this protein is associated with increased risk of carcinogenesis and increased sensitivity to methylating agents (6). Abnormal MGMT expression causes O6-methylguanine to accumulate in cellular DNA (7). MGMT prevents mutagenesis and malignant transformation, and also provokes resistance to chemotherapy with alkylating agents in cancer patients (6,8). It is shown that methylation of the MGMT gene promoter is a common event in sporadic colorectal cancer adjacent to the normal-appearing colorectal mucosa (9).

The DNA methyltransferase (DNMT1) [NCBI Gene ID: 1786] catalyzes the addition of methyl groups to cytosine bases in DNA, and it is found in most, if not all, cells of mammals (10). DNA methylation plays a crucial role in transcriptional regulation and chromatin remodeling of mammalian cells (11). Both DNA hypomethylation and/or regional DNA hypermethylation have been well documented in various tumors (12). Besides, over-expression of DNMT1 has been detected in several human cancers (13,14).

We hypothesized that amino acid substitution polymorphisms of MGMT and DNMT1 genes could be mediators of field conversion to malignancy of the colon mucosa. To test this hypothesis, we studied five polymorphisms of MGMT (Pro58Ser, Leu84Phe, Arg128Gln, Ile143Val, Gly160Arg), and three of DNMT1 (Ile311Val, Ala147Gly, His97Arg) in the 208 patients with sporadic colorectal cancer, as well as in the 213 healthy individuals

PATIENTS and METHODS

The present study included 208 sporadic colorectal cancer patients and 213 cancer free controls that were recruited between September 2003 and December 2007 at the Research Institute for Gastroenterology and Liver Diseases in Taleghani Hospital (Table 1).

Table 1. Clinicopathological characteristics of patients*

Characteristic	
Mean age at diagnosis (Years)	41.93
Male/Female	83/117
Tumor site	
Colon	123†
Rectum	77
Tumor stage	
0	0
I	1
II	24
III	31
IV	2
Tumor grade	
Undetermined	0
Low	32
Intermediate	41
High	12
Cigarette smoking	
Non smoker	114
Smoker	68
Positive family history	112

* None of cases had high energy intake or alcohol consumption.
† Frequency

All subjects were genetically-unrelated Iranian patients whose diagnoses were confirmed to be sporadic colorectal cancer based upon histopathologic exams. Cancer-free controls were

Table 2.Primer sequences for all selected polymorphisms

SNP	Forward Primer	Reverse Primer	Sequencing Primer
1 Pro58Ser	AGTGCCGTGGAGGTCCCAG*	GGGCTGGTGGAAATAGGCA	CTGTGCACTGCATCA
2 Leu84Phe	TCGAAGAGTTCCCCGTGCC	CCAAAGGAAACACCGCAGATG*	TTCCCCGTGCCGGCT
3 Arg128Gln	AGCAATTAGCAGCCCTGGCA	TGCTTGCGCCATGAGAACTC*	CAACCCCAAAGCCGC
4 Ile143Val	*CCCCAAAGACCTCGTTGTCC	ATTCCTTCACGGCCAGTCCTC	ACCACTCTGTGGCACG
5 Gly160Arg	CGTGCCACAGAGTGGTCTGC	CATGGGCCAGAAGCCATTC*	CCGTGGGCAACTACTC
6 Ile311Val	GTATCTGTTCACCCTGCAGAGCT	TCATCCTCGTCTTTTTCATC*	TGAAAAAGTAAATCCAC
7 Ala147Gly	CCCCAAACCCCTTTCCAA*	TGGGCAACACAGTGAGACTCC	CATCTGCTCTTACGCTT
8 His97Arg	GCTTGGTTCCCGTTTTCTAGAC*	GGGCTACCTGGCTAAAGTCAAA	CCTTGGAGAACGGTG

* Biotinylated (Biotin group is added to 5' primer)

randomly selected from individuals referred to Taleghani hospital; these control subjects had no history of cancer and were frequency-matched to the cases by age within five years and sex.

Five milliliters of venous blood was collected in vacuum tubes containing EDTA and stored at 4˚C. Genomic DNA was extracted within one week of sampling using a standard phenol-chloroform extraction method (15).

The loci for the eight non-synonymous SNPs (five of MGMT and three of DNMT1) were amplified by polymerase chain reaction (PCR). The SNPs were chosen from three databases: Ensemble, Pubmed and Hapmap.

After SNP selection we designed specific PCR and pyrosequencing primers for each SNP (Table 2). Technically, we needed three primers for each SNP: forward, reverse (one of which was biotinylated), and Sequencing primers.

PCR was performed in a final volume of 25 µL containing 100 ng of template DNA, 2.5 µL of 10X PCR buffer, 1 U of Taq-DNA-polymerase, 200 µmol/L of dNTPs and 400 nmol/L of primers of which one was biotinylated. The PCR program consisted of an initial denaturation step at 95˚ C for 5 min followed by 35 cycles of denaturation at 95˚ C for 30 s, annealing at 55-67˚ C (depending on the loci) for 30 s, extension at 72˚ C for 40 s, and a final step of elongation at 72˚ C for 10 minutes.

We needed three primers for each SNP of which two were used for amplification by PCR and one was used for Pyrosequencing. First we amplified the target region by PCR. This done, the single stranded DNAs (ssDNAs) were selected by using streptavidin-coated beads (GE Healthcare)

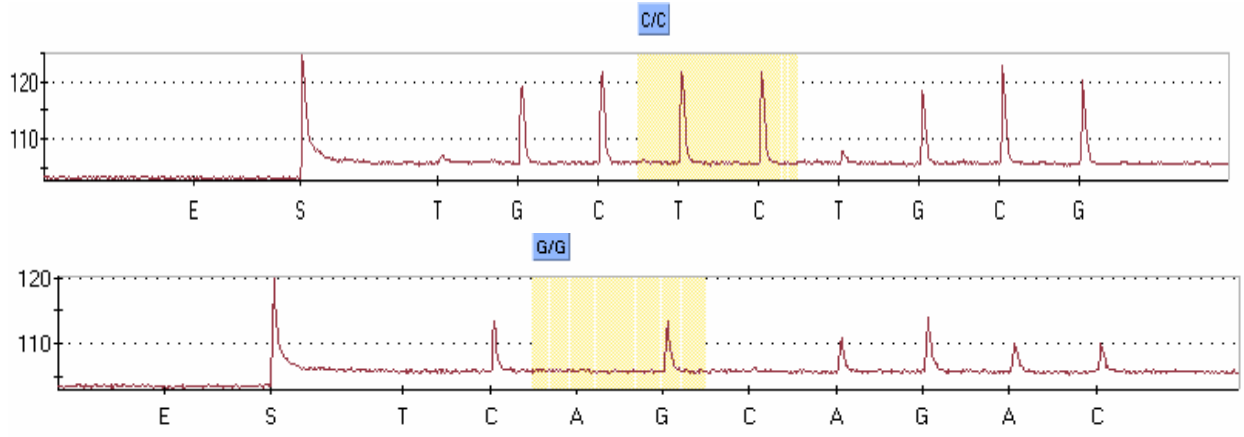

Figure 1. Pyrograms of Arg128Gln and Gly160Arg polymorphisms of MGMT gene in order

Table 3. Genotype frequencies and association of MGMT and DNMT1 genes with colorectal cancer assuming a codominant model

	Controls			Cases						
							+/– vs +/+		–/– vs +/+	
	+/+	+/–	–/–	+/+	+/–	–/–	OR (95% CI) *	P-value	OR (95% CI)	P-value
MGMT SNP										
Pro58Ser	98	74	28	89	81.4	30.6	1.21(0.77-1.89)	0.39	1.18(0.63-2.22)	0.58
Leu84Phe	60.8	140	0	40	160	0	1.23(0.87-2.36)	0.14	-	-
Arg128Gln	186	10	4	148	44	8	5.53(2.58-7.16)	0.005	2.51(0.67-10.13)	0.13
Ile143Val	146	52	2	125	74	1	1.68(1.07-2.63)	0.02	0.59(0.02-8.38)	0.66
Gly160Arg	176	14	10	124	30	46	3.04(1.48-6.31)	0.005	5.37(2.75-10.65)	0.005
DNMT1 SNP										
Ile311Val	148	50	2	140	58	2	1.10(0.60-2.13)	0.68	0.95(0.04-2.48)	0.96
Ala147Gly	92	80	28	90	80	30	1.02(0.42-2.68)	0.92	1.10(0.60-2.13)	0.76
His97Arg	148	34	18	138	34	28	1.09(0.34-2.22)	0.67	1.34(0.67-3.12)	0.37

* Odds Ratio (95% Confidence Interval)

and washing. These ssDNAs were genotyped in polymorphism locus by sequencing primer and pyrosequencer (PSQ 96MA).

Pyrosequencing analysis of PCR products was performed using the manufacturer's recommended protocol (16,17). The polymorphic positions were analyzed using a PSQ 96MA system, SNP software and SNP PyroGold reagent kits. Then, the peaks were evaluated according to the expected pattern by referring to the dispensation order. Genotyping calls were determined automatically using the PSQ 96MA 2.1.1 Software version 1.0 provided by the manufacturer (Figure 1).

Allele frequencies were calculated by counting alleles. Goodness of fit between observed and estimated genotype frequencies was determined by chi-square test, according to the Hardy–Weinberg equilibrium (HWE). The observed genotype frequencies were compared with those calculated from Hardy-Weinberg equilibrium theory ($p2+2pq+q2=1$ where p is the frequency of the variant allele and $q=1-p$). In this study, we hypothesized that the presence of the polymorphic allele might be associated with a higher risk of colorectal cancer. However, because it was unclear whether the polymorphic allele had a dominant, recessive or gene-dosage effect, statistical modeling was performed on the relative risk of the mutant/mutant genotypes or the wild/mutant genotypes against the wild/wild genotypes respectively. The odds ratio (OR) and 95% confidence interval (CI) were calculated to estimate the relative risk. All statistical tests were two-sided and performed with Statistical Package for Social Science V.10.0 (SPSS, Chicago, IL).

RESULTS

We genotyped the study participants for five non-synonymous polymorphisms in MGMT gene: Pro58Ser, Leu84Phe, Arg128Gln, Ile143Val, and Gly160Arg; and three in DNMT1 gene: Ile311Val, Ala147Gly, His97Arg. Of these eight amino acid substitution variants, two SNPs (Pro58Ser and Leu84Phe) were located within exon 5, one within exon 6 (Arg128Gln), two within exon 7 of MGMT (Ileu143Val and Gly160Arg) and three on exon 4 of DNMT1 gene (Ile311Val, Ala147Gly and His97Arg). The frequencies of the genotypes of all studied polymorphisms are shown in Table 3. When the non-synonymous polymorphisms of 213 healthy individuals enrolled in the study were pyrosequenced, we found the frequencies in agreement with PubMed reports and in Hardy-Weinberg equilibrium. The frequencies of

Table 4. Interactions of selected polymorphisms with age, family history and cigarette smoking

	Cases/controls	OR (95%CI) *	P-value†	Cases/controls	OR (95%CI)	P-value
Age ranges (years)						
Arg128Gln		Arg/Arg-Arg/Gln			Gln/Gln	
50-69	122/138	0.89 (0.77-1.89)	0.498	18/16	1.05 (0.53-2.40)	0.910
70-95	48/34	1.43 (0.85-2.35)	0.150	12/12	0.93 (0.41-2.44)	0.886
Gly160Arg		Gly/Gly-Gly/Arg			Arg/Arg	
50-69	115/134	1.06 (0.41-1.07)	0.732	41/10	1.02 (0.83-3.93)	0.957
70-95	39/56	0.86 (0.53-1.33)	0.518	15/4	0.94 (0.54-8.3)	0.919
Positive Family History						
Arg128Gln		Arg/Arg-Arg/Gln			Gln/Gln	
	98/44	1.15 (0.67-1.64)	0.557	18/14	0.67 (0.28-1.48)	0.296
Gly160Arg		Gly/Gly-Gly/Arg			Arg/Arg	
	89/35	1.32 (0.77-2.25)	0.283	23/23	0.34 (0.25-1.05)	0.001
Cigarette smoking						
Arg128Gln		Arg/Arg-Arg/Gln			Gln/Gln	
	84/56	0.89 (0.55-1.44)	0.629	30/12	1.49 (1.49-3.32)	0.283
Gly160Arg		Gly/Gly-Gly/Arg			Arg/Arg	
	80/61	0.78 (1.40-2.39)	0.282	34/7	2.90 ((1.15-7.61)	0.012

* Odds Ratio (95% Confidence Interval)

† *P* for the test of interaction between the trend in risk for age and the genotype.

polymorph alleles were compared between colorectal cancer patients and healthy individuals.

There was found no significant relationship between DNMT1 polymorphisms and colorectal cancer, but significant associations were found for Arg128Gln and Gly160Arg of MGMT gene (Table 3). Carriers of these two polymorphisms had a significantly increased risk of sporadic colorectal cancer compared with healthy individuals. Homozygous carriers of the Gly160Arg polymorphism had a significantly increased risk of sporadic colorectal cancer compared with carriers of the major allele (for the recessive model that combines as reference group individuals homozygous for the major allele and heterozygous). Heterozygous carriers of Arg128Gln and Gly160Arg polymorphisms had a significantly increased risk of sporadic colorectal cancer compared with carriers of the major allele.

The interaction of these two polymorphisms with age, positive family history of colon cancer, and cigarette smoking was determined, and we found that only homozygous polymorph (Arg/Arg) Gly160Arg in smoker patients increased the risk of colorectal cancer in this population (Table4).

DISCUSSION

It has been reported that the frequency and degree of DNA hypermethylation would increase in a group of cancers such as lung cancer and hepatocellular carcinoma (18,19). It has also been reported that DNMT1 mRNA overexpression correlates significantly with CpG island methylator phenotype in gastric and colorectal cancers (20,21). Because MGMT plays an essential role in repairing DNA damage caused by environmental alkylating chemicals, we were interested to determine whether we could see any obvious changes in the properties of colorectal cancers (CRCs). According to some studies, MGMT can be hypermethylated in a number of tumors, including colorectal carcinomas (22).

In this study, we explored five non-synonymous polymorphisms in MGMT gene that were relevant to DNA repair pathways against alkylating agents: Pro58Ser, Leu84Phe, Arg128Gln, Ile143Val and Gly160Arg. Polymorphisms were generally selected according

to prior data on functional effect or reports of association to malignancies to increase the possibility of positive findings.

Both the MGMT Leu84Phe and MGMT Ile143Val variant alleles have previously been associated with either significantly increased (23-25) or decreased risk (26) of various types of human cancer. Ile143Val is one of the most common non-synonymous SNPs in MGMT. This amino acid substitution may change the function of the protein and, therefore, cause diseases such as cancer. Ile143Val that is linked with Lys178Arg may modulate the protein's function because residue 143 is close to the conserved alkyl group acceptor, codon 145, in the active site of MGMT (27). The Ile143Val has previously been related to increased lung cancer risk in two small studies (28). Three-dimensional structural modeling of MGMT revealed that the Leu84Phe, Ile143Val and 145 cysteine alkyl residues pack in a hydrophobic region with the LXXLL motif (28), suggesting that our objective two polymorphisms (Leu84Phe, Ile143Val) may affect both the MGMT functions, inhibit estrogen receptor cell proliferation and DNA repair.

Most of other studies found no significant functional difference between the variant alleles and the wild type (29-31), which to some extent support the overall null association of the two MGMT polymorphisms with colorectal cancer risk in our study. There was no significant association between Ile143Val and inclination toward CRC. The Ile143Val polymorphism does not affect DNA repair capacity (32). The frequency of the MGMT Ile143Val allele was similar to that reported in two European studies (32,33).

Arg128Gln and Gly160Arg polymorphisms had a significantly increased risk of sporadic colorectal cancer compared with carriers for the major allele. Arg128Gln is crucial for DNA binding and Gly160Arg is important for the acceptor reaction of the protein with O6-benzylguanine (34, 35). We confirmed a significant relationship between MGMT heterozygote Arg128Gln polymorphism and colorectal cancer.

Variability in the prevalence of the Gly160Arg has been observed between studies of different cohorts of the same competition (36). Imai et al described the presence of this allele in 3 of 28 healthy Japanese controls (37). Gly160Arg polymorphisms were not detected in studies examining allelic prevalence in Caucasians (38,39), African Americans (39), and Asians [including Chinese (38) and a small cohort of Japanese (39)]. In the present study, we confirmed the existence of the Gly160Arg allele in colorectal cancer patients more than controls. The data from the present study suggested that the Gly160Arg allele was associated with a high risk of colorectal cancer; however, for an efficient risk assessment analysis, a much larger study with sufficient power should be conducted. There was no previous research on Pro58Ser of MGMT and Ile311Val, Ala147Gly, His97Arg of DNMT1 and colorectal cancer and we found no relationship between these polymorphisms and colorectal cancer in this research. Moreover, this study provided the first report on the association between colorectal cancer and DNMT1 polymorphisms among Iranian patients. Two polymorphisms, Arg128Gln and Gly160Arg, showed significant association in our studied population, which suggests that common variants in DNA repair genes affects the etiology of sporadic colorectal cancer.

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