RESEARCH COMMUNICATION

Effects of Amino Acid Substitution Polymorphisms of two DNA Methyltransferases on Susceptibility to Sporadic Colorectal Cancer

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Abstract

Background and Aim: The present study was designed to consider whether amino acid substitution polymorphisms in O^6-methylguanine-DNA methyltransferase (MGMT) and DNA methyl transferase 1 (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 two groups. Results: The calculated odds ratios indicated no association between DNMT1 and colorectal cancer. However, there was a significant association between two polymorphisms in MGMT with sporadic colorectal cancer: Arg128Gln (OR, 5.53) and Gly160Arg (OR, 3.04) . Conclusions: These findings could be indicative of factors contributing to high occurrence of Iranian colorectal cancer patients.

Key words: MGMT - DNMT1 - amino acid substitution polymorphisms - colorectal cancer - Iran

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Introduction

Colorectal carcinoma 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 (Leddin et al., 2004). Genomic stability is maintained by DNA repair genes in dividing cells. An individual's capacity to repair DNA is genetically determined and the result of combinations of multiple genes with different activities is in consideration. Single nucleotide polymorphisms (SNPs) are common allelic variants occurring around once per every 500 to 1,000 base pairs in the human genome (Sauer et al., 2000). 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 susceptibility to diseases like cancer (Miao et al., 2003; Hemminki et al., 2005). One procedure of DNA repair is methylation of the O^6-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 O^6-methylguanine during DNA replication may cause a G:C to A:T transition (Aquilina et al., 1992), because O^6-methylguanine residues preferentially pair with thymine during DNA synthesis. The O^6-methylguanine DNA methyltransferase (MGMT; MIM# 156569; GDB: 125264) gene is responsible for repairing alkylation DNA damage, and removes alkylating groups at the position O^6 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 carcinogenesis risk and increased sensitivity to methylating agents (Gerson, 2004). Abnormal MGMT expression causes O^6-methylguanine to accumulate in cellular DNA (Ishibashi et al., 1994). MGMT prevents mutagenesis and malignant transformation, and also provokes resistance to chemotherapy with alkylating agents in cancer patients (Esteller and Herman, 2004; Gerson, 2004). It is shown that methylation of MGMT gene promoter is a common event in sporadic colorectal cancer adjacent to normal-appearing sporadic colorectal mucosa (Shen et al., 2005).

The DNA methyl trasferase (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 the mammals (Trasler et al., 1996). DNA methylation plays a crucial role in transcriptional regulation and chromatin remodeling of mammalian cells (Kondo et al., 2000). Both DNA hypomethylation and/or regional DNA hypermethylation have been well documented in various tumors (Okano et al., 1998). Besides, over-expression of DNMT1 has been detected in several human cancers (Kanai et al., 2001; Saito et al.,

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Table 1. Characteristics of Cases and Controls

Characteristic		Cases	Controls
Mean age (years)		41.9	45.1
Sex: Male/Female		83:117	81:119
Tumor site	Colon	123	-
	Rectum	77	-
Tumor stage	I	1	-
	II	24	-
	III	31	-
	IV	2	-
Tumor grade	Low	32	-
	Intermediate	41	-
	High	12	-
Smoking	Yes/No	68:114	63:123
Positive family history		112	27

None of cases or controls had high energy intake or alcohol consumption

2001).

We hypothesized that amino acid substitution polymorphisms of MGMT and DNMT1 genes could be mediators of field cancerization 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

Materials & Methods

Subjects

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 Center of Gastroenterology and Liver Diseases (RCGLD) in Taleghani hospital (Table 1). All subjects were genetically-unrelated Iranian Patients whose diagnoses were confirmed to be sporadic colorectal cancer based upon histopathologic exams. Cancer-free controls were 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.

DNA extraction

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 (John et al., 1991).

SNP selection and PCR amplification

The loci for the eight non-synomymous SNPs (five of MGMT and three of DNMT1) were amplified by polymerase chain reaction (PCR). The SNPs were chosen from three databases: www.ensemble.org, www.genome.ucsc.edu and www.ncbi.nlm.nih.gov.

After SNP selection we designed specific PCR and pyrosequencing primers for each SNP (Table2). Technically we needed three primers for each SNP: Forward, reverse that one of them is biotenylated and sequencing primers.

PCR reactions were 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 that one of them were biothenylated . 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 (dependent 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.

Genotyping by Pyrosequencing

We need 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 and then by using Streptavidine-Coated Beads (GE Healthcare) and washing, the single stranded DNAs (ssDNA) was selected. These ssDNAs were genotyped in polymorphism locus by sequencing primer and pyrpsequencer (PSQ 96MA).

Pyrosequencing analysis of PCR products was performed using the manufacturer's recommended protocol (Pettersson et al., 2003; Ronaghi, 2003). The polymorphic positions were analyzed using a PSQ 96MA system, SNP Software and SNP PyroGold Reagent Kits. Then, the run 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).

Statistical analysis

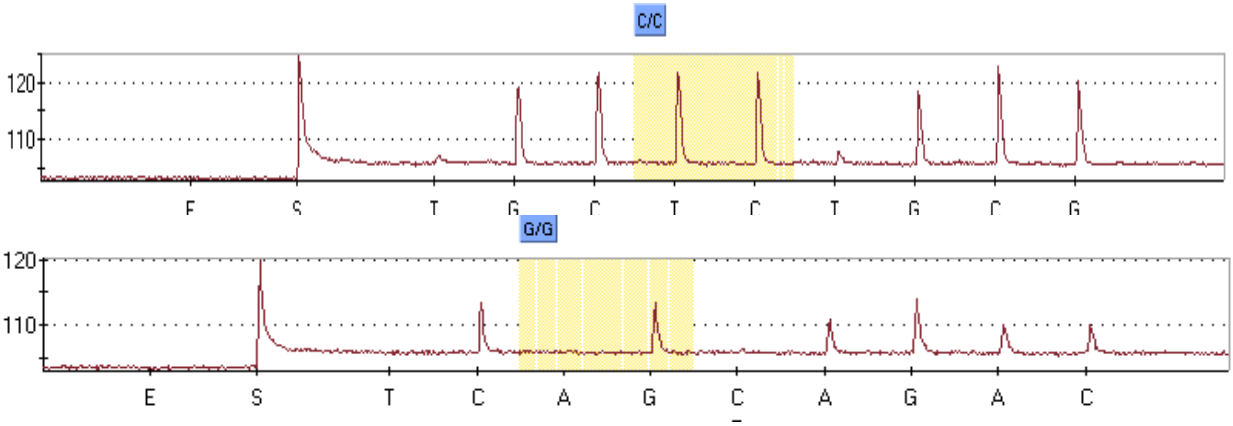

Figure 1. Pyrograms of Arg128Gln and Gly160Arg Polymorphisms of the MGMT gene in order

Table 2. Primer Sequences for all Selected Polymorphisms

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

*Biotenylated (Biotin group added to the 5' primer)

Table 3. Genotype Frequencies and Association of MGMT and DNMT1 Genes with Colorectal Cancer Assuming a Codominant Model

	Controls			Cases			+/– vs +/+		–/– vs +/+	
	+/+	+/–	–/–	+/+	+/–	–/–	OR	P-value	OR	P-value
MGMT SNP										
Pro58Ser	98	74	28	89	81	31	1.21	0.390	1.18	0.582
Leu84Phe	61	140	0	40	160	0	1.23	0.138	-	-
Arg128Gln	186	10	4	148	44	8	5.53	<0.005	2.51	0.126
Ile143Val	146	52	2	125	74	1	1.68	0.017	0.59	0.663
Gly160Arg	176	14	10	124	30	46	3.04	<0.005	5.37	<0.005
DNMT1 SNP										
Ile311Val	148	50	2	140	58	2	1.10	0.681	0.95	0.955
Ala147Gly	92	80	28	90	80	30	1.02	0.919	1.10	0.762
His97Arg	148	34	18	138	34	28	1.09	0.674	1.34	0.369

Allele frequencies were calculated by counting alleles. Goodness of fit between observed and estimated genotype frequencies according to the Hardy-Weinberg equilibrium (HWE) was determined by chi-square test. 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 ORs and 95% CIs 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 (Pro58Ser, Leu84Phe, Arg128Gln, Ile143Val, Gly160Arg) and three (Ile311Val, Ala147Gly, His97Arg) of DNMT1 genes. Of these eight amino acid substitution variants, two SNPs (Pro58Ser and Leu84Phe) were located within exon 5, Arg128Gln within exon 6 , two SNPs Ileu143Val and Gly160Arg within exon 7 of MGMT and Ile311Val, Ala147Gly, His97Arg on exon 4 of DNMT1 gene. The frequencies of the genotypes of all studied polymorphisms are shown in Table 3. When these eight non-synomymous 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 polymorph alleles were compared between colorectal cancer patients and healthy individuals (see Figure 2).

There was found no significant relationship between DNMT1 polymorphisms and colorectal cancer but significant associations have been found for Arg128Gln and Gly160Arg of MGMT gene. 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

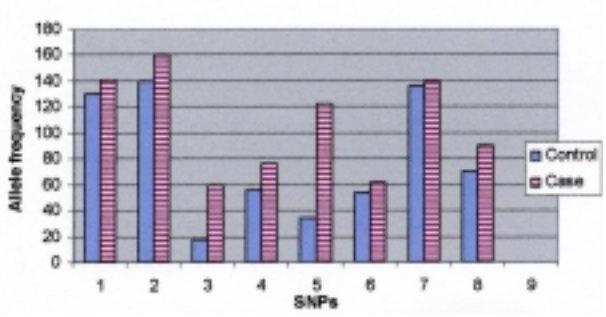

Figure 2. Frequencies of Polymorph Alleles in Colorectal Cancer Patients (Case) and Healthy Individuals (Control). The x axis show polymorphisms, 1: Pro58Ser(OR:1.09,CI:95%,P:0.529) , 2 : Leu84Phe (OR:1.14, CI:95%, P:0.324), 3: Arg128Gln (OR:0.30, CI:95%, P<0.005), 4: Ile143Val (OR: 1.36, CI:95%, P:0.106), 5: Gly160Arg (OR:0.28, CI:95%, P<0.005),6: Ile311Val (OR:1.15, CI:95%,P:0.487) , 7: Ala147Gly (OR: 1.03, CI: 95%,P: 0.835) and 8: His97Arg (OR:1.29, CI:95%,P:0.148). With Arg128Gln (OR:0.30,CI:95%,P<0.005) and Gly160Arg (OR:0.28, CI:95%,P<0.005) there was a meaningful difference for polymorphic allele occurrence between case and controls in these two polymorphisms

Table 4. Interactions with Age, Family History and Cigarette Smoking of Selected Polymorphisms

	Cases/controls	OR	P-value	Cases/controls	OR	P-value*
Age ranges (y)						
Arg128Gln		Arg/Arg-Arg/Gln			Gln/Gln	
50-69	122/138	0.89	0.498	18/16	1.05	0.910
70-95	48/34	1.43	0.150	12/12	0.93	0.886
Gly160Arg		Gly/Gly-Gly/Arg			Arg/Arg	
50-69	115/134	1.06	0.732	41/10	1.02	0.957
70-95	39/56	0.86	0.518	15/4	0.94	0.919
Positive Family History						
Arg128Gln		Arg/Arg-Arg/Gln			Gln/Gln	
	98/44	1.15	0.557	18/14	0.67	0.296
Gly160Arg		Gly/Gly-Gly/Arg			Arg/Arg	
	89/35	1.32	0.283	23/23	0.34	0.001
Cigarette smoking						
Arg128Gln		Arg/Arg-Arg/Gln			Gln/Gln	
	84/56	0.89	0.629	30/12	1.49	0.283
Gly160Arg		Gly/Gly-Gly/Arg			Arg/Arg	
	80/61	0.78	0.282	34/7	2.90	0.012

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

sporadic colorectal cancer compared with carriers for the major allele (for the recessive model that combines as reference group individuals homozygous for the major allele and heterozygous: OR, 5.37; 95% CI???, , P < 0.005). Heterozygous carriers of Arg128Gln and Gly160Arg polymorphisms had a significantly increased risk of sporadic colorectal cancer compared with carriers of the major allele. For Arg128Gln and Gly160Arg polymorphisms the odds ratio and p-value were OR,5.53 ; 95% CI , P < 0.005, and OR,3.04 ; 95% CI ???, P < 0.005 respectively.

The interaction of these two polymorphisms and age, positive family history of colon cancer, and cigarette smoking were determined, and we found that only homozygous polymorph (Arg/Arg) Gly160Arg in smoker patients (OR= 0.34,CI=95% ????,P<0.005) demonstrated increased risk of colorectal cancer in this population (Table 4).

Discussion

It has been reported that the frequency and degree of DNA hypermethylation was increased in other groups of cancers such as lung cancer, hepatocellular carcinoma, hepatitis and liver cirrhosis compared with the normal mucosa (Kanai et al., 1998; 2000). It was reported that DNMT1 mRNA overexpression correlates significantly with CpG island methylator phenotype in gastric and colorectal cancers (Saito et al., 2001; Etoh et al., 2004). Because MGMT plays an essential role in repairing DNA damage caused by environmental alkylating chemicals, we were interested in determining whether we could see any obvious changes in the properties of colorectal cancers (CRCs). A number of studies showed that MGMT can be hypermethylated in tumors, including colorectal tumors (Eads et al., 2001).

We have explored Pro58Ser, Leu84Phe, Arg128Gln, Ile143Val and Gly160Arg (non-synomymous polymorphisms) in MGMT gene that are relevant to DNA repair pathways against alkylating agents. 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 (Kaur et al., 2000; Cohet et al., 2004; Ritchey et al., 2005) or decreased risk (Huang et al., 2005) 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, which 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 (Chueh et al., 1992). The Ile143Val has previously been related to increased lung cancer risk in two small studies (Tranah et al., 2006). 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(Tranah et al., 2006), suggesting that our objective two polymorphisms (Leu84Phe, Ile143Val) may affect both the MGMT functions, inhibit estrogen receptor cell proliferation and DNA repair.

Most other studies found no significant functional difference between the variant alleles and the wild type (Inoue et al., 2000; Mijal et al., 2004; Savas et al., 2004), 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 of CRC. The Ile143Val polymorphism does not affect DNA repair capacity (Ma et al., 2003). The frequency of the MGMT Ile143Val allele was similar to that reported in two European studies (Egyhazi et al., 2002; Ma et al., 2003).

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 are important for the acceptor reaction of the protein with O^6-benzylguanine (Kanugula et al., 1995; Xu-Welliver and and Pegg, 2000). We confirmed the 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 (Tajinder et al., 2000). Imai et al (1995) described the presence of this allele in 3 of 28 healthy Japanese controls. Gly160Arg polymorphisms were not detected in studies examining allelic prevalence in Caucasians (Deng et al., 1999), African Americans (Wu et al., 1999), and Asians [including Chinese (Deng et al., 1999) and a small cohort of Japanese (Wu et al., 1999)]. 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 suggest that the Gly160Arg allele is 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. It is important report because this is the first report on the association between colorectal cancer and DNMT1 polymorphisms among Iranian patients. Two polymorphisms, Arg128Gln and Gly160Arg, show significant association in our studied population, that suggest that common variants in DNA repair genes have effect in the etiology of sporadic colorectal cancer.

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