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Methylation profile of promoter region determined level of *MGMT* mRNA expression in colorectal cancer

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Abstract

O6-methylguanine DNA methyltransferase (MGMT) is a DNA repair enzyme that is important for colorectal to stay protected against DNA mutagenesis. The role of MGMT reduction or loss in colorectal tumorigenesis is complex but numerous studies have documented methylation of this gene can be regarded as an event in colorectal cancer. The aim of this study is to demonstration of correlation between methylation profile in promoter region of *MGMT* with MGMT expression level, CRC occurence and with demographic and clinocopathological characteristics of CRC. Methylation profile in promoter region of *MGMT* was assayed by methylation-specific polymerase chain reaction (MS-PCR) and *MGMT* gene expression was performed by real time quantitative PCR (qPCR). RNA from embedded paraffin sections of colorectal tissue (in 70 sporadic colorectal tumors as well as adjoining and normal tissue specimens) was reverse transcribed, quantified and analyzed. Aberrant promoter methylation of *MGMT* gene was detected in 23 (32.9%) of 70 tumor samples and in 5 (7.1%) adjacent normal tissues. Thus, aberrant promoter methylation of *MGMT* is significantly correlated with CRC occurance. Aberrant promoter methylation was not found significantly associated with tumor stage and other clinocopathological and demographic characteristics. *MGMT* expression level in tumor tissues was 7.2-fold less than normal adjacent tissues. In conclusion, this study has identified aberrant promoter methylation of *MGMT* was significantly correlated with CRC, because aberrant promoter methylation effects on *MGMT* expression. Our assay demonstrated *MGMT* can be as a potential biomarker for CRC as diagnostic, prognostic and therapeutic targets in the future.

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Introduction

Colorectal cancer (CRC) is the third most common cancer in men cases and the second in women with about 608000 deaths in the worldwide. CRC rates are about 2 to 5 times higher in the developed countries in comparison to the developing countries which may be attributable to a range of variations in a disparate set of risk factors and diagnostic practices (Notani, 2001; Jemal *et al.*, 2011). Lowest incidence rates are observed in Africa and highest ones are found in Western societies. In USA, data from migration population studies revealed that some ethnic groups are showing increased CRC incidence rate while they are migrating from low-risk to high-risk areas (Grulich *et al.*, 1995; Lee *et al.*, 2007). Despite genetic variation, the epidemiological data strongly suggest a role of environmental and lifestyle factors deeply contributing to the etiology of CRC. Significant lifestyle risk factors are changes in dietary habits, from a moderate to a Western like enriched diet with high unsaturated fats and red meat, high intake of alcohol, and smoking. It is well accepted that environmental and dietary factors greatly influence epigenetic events including hypermetylation.

DNA methylation is a significant regulator of gene transcription, and its role in carcinogenesis has become a topic of considerable interest in the last few years. DNA cytosine methylation has been widely studied, with investigations often focusing on the methylation level of CpG dinucleotides in promoter regions that usually have higher concentrations of CpGs, known as CpG islands (Jones and Baylin, 2002). The methylation of normally unmethylated CpG islands in the promoter regions of DNA repair genes is correlated with a loss of expression of these genes (Baylin and Herman, 2000; Herman and Baylin, 2003), which often occurs in the early stages of colorectal cancer (CRC) development (Derks., *et al*, 2006; Esteller, 2000; Frigola., *et al*, 2005).

A few investigations conducted on cancer-associated genes including cyclin-dependent kinase inhibitor 2A (*P16*), E-cadherin (*CDH1*), O^6-methyl guanine methyl transferase (*MGMT*) and Estrogen Receptor-α (*ESR-α*) genes, have been found to harbor hypermethylated regulatory sequences that lead to gene silencing in CRC (Mehrzad *et al.*, 2014; Yu *et al.*, 2005; Wani *et al.*, 2013). However, numbers of studies to evaluate methylation profile of MGMT in promoter region is low in CRC and further investigation is necessary.

O6-methylguanine-DNA methyltransferase (MGMT) is a DNA repair enzyme removing alkyl groups from the O^6 position of guanine (Sidhu *et al.*, 2010). O^6-alkylguanine adducts cause damage by mispairing with thymine during replication leading to G:C to A:T transitions (Gerson, 2004). *MGMT* promoter hypermethylation and epigenetic silencing trigger often occur as early events in carcinogenesis (Shima *et al.*, 2011). Thus, *MGMT* was selected and studied in this investigation. The present study was thus conducted to study the role of methylation profile of promoter region of *MGMT* gene in CRC in Iranian population and its correlation with the clinicopathological variables of CRC.

Here we used methylation-specific polymerase chain reaction (PCR) (MS PCR) to investigate *MGMT* methylation to distinguish methylated from unmethylated cytosines. MS PCR is very sensitive to detect one methylated cytosine in 1000, and the primers used are highly specific and cost effective (Herman *et al.*, 1996). Also we used real -time quantitative PCR, that it is a very precision method, to assay *MGMT* mRNA expression. In fact, the aim of this study was to evaluate the effect of methylation status on *MGMT* mRNA expression and correlation between these cases and CRC risk.

Materials and methods

Patient population and tissue samples

Seventy patients with histologically confirmed colorectal cancer (primary sporadic CRC) were examined in the present study. All patients underwent colorectal resection primarily at Neyshabur Medical Science Faculty Hospital (Neyshabur, Iran) from January 2008 to February 2013. No patient had received preoperative treatment in the form of radiation or chemotherapy. Patients

who had other malignant disease except CRC were excluded. This study was approved by the IAU-Neyshabur and Neyshabur Medical science Faculty. All the tissue samples were collected within 10 minutes of resection. Tumor samples were divided into two parts; one part was sent for histopathological diagnosis and staging and the other half was stored as formalin fixed paraffin embedded (FFPE) as well as adjacent normal samples (normal non tumorous healthy tissue at >2 cm distance from the tumor) (Li *et al.*, 2012). Histopathological grades and clinical staging were evaluated according to the standard criteria (Edge *et al.*, 2010). Only histopathologically confirmed cases were included for further molecular analysis.

DNA and RNA extraction from tissue samples

Genomic DNA was extracted 3 times from 70 paired normal background and tumor FFPE tissue specimens with the AccuPrep Genomic DNA Extraction Kit (BioNEER, Cat.NO.: K-3032, Korea).
The most important consideration in generating useful data with real-time q-PCR is the quality of the isolated RNA. RNA isolated 5 times with the Jena Bioscience kit (Cat.-No. PP-210xs, Germany).
Before all extractions FFPE tissue specimens was deparaffinized by xylen. Extracted DNA quantity/quality was checked spectrophotometrically and gel electrophoresis and stored for further use at -20°C.

Bisulfite modification and methylation- specific polymerase chain reaction (MS PCR)

Aberrant DNA methylation in the CpG islands in the promoter region of the *MGMT* in 70 patients of CRC was determined by chemical modification of genomic DNA with sodium bisulfite followed by MS PCR. Genomic DNA was treated with sodium bisulfate as reported by Herman *et al* (1996). Briefly, 2 µg of genomic DNA was denatured in 50 µl NaOH (final concentration, 0.2 M) for 10-15 min at 50 °C. Thirty microliters of 10 mM hydroquinone (Fluka) and 520 µl 3 M sodium bisulfite (Fluka) at pH 5, freshly both prepared, were added and mixed, and the samples were incubated at 50 °C for 16 h. The unmethylated cytosine was converted to thymine, whereas methylated cytosine remains unchanged.

The primer sequence of MGMT was based on the previous report and is listed in Table 1. Briefly, the PCR mixture containing 5 µl of 5X reaction buffer (100 mmol/L Tris–HCl {pH8.3}, 500 mmol/L KCl, 15 mmol/L MgCl2), 10 µl of modified DNA, 10 mmol of each primer, 1 µl deoxynucleotide triphosphates (200 µmol/L each, final concentration), and 1 U of hot start Taq DNA polymerase (Promega, USA) was adjusted to final volume of 25 µl using sterile water. The cycling conditions consisted of an incubation period at 95°C for 15-min 40 cycles of denaturation at 94°C for 30 s and 58°C for 30 s , extension at 72°C for 30 s and final extension at 72°C for 10 min. Positive. Blood Sample of normal human DNA was used as the control for unmethylated alleles. In vitro methylated DNA from placenta with SssI methyltransferase was used as a positive control for methylated reaction. Distilled water was used as a negative control.

PCR products were electrophoresed on 2.5% agarose gels and visualized by ethidium bromide staining. Samples were scored as methylated when there was a clearly visible band on the gel with the methylation-specific primers. The sizes of PCR product in this study were 81 bp for methylation and 93 bp for unmethylation respectively. All samples were examined by one experimenter who was unaware of the patient's clinical features.

Real-time quantitative PCR (qPCR)

Two-step procedure was conducted in this study. cDNA was prepared according to manufacturer's instructions of kit (AccuPower RoketScript RT PerMix) from BioNEER company of korea. q PCR was performed in triplicates with the Applied Biosystems step one Real-Time PCR System, using SYBR® Green PCR Master Mix (Part number 4309155) in comparative Ct method {ΔCt = Ct(main target cDNA) – Ct (refrence cDNA) and ΔΔCt = ΔCt (test cDNA) - ΔCt (calibrator cDNA) }. The primers were used for *MGMT* and *GAPDH* as an internal control, are shown in table 2 based on other

references (Tanaka *et al.*, 2003). The PCR reactions were set up in microtubes in a volume of 20 µl. The reaction components were 2.0µg of cDNA synthesized as above, 10 µl of 2× SYBR Green PCR Master Mix (Applied Biosystems), and 0.05 µM of each pair of oligonucleotide primers.The program was as follows: initial melting for 1 min at 96°C, cycles consisting of melting for 30 sec at 96°C, annealing for 30 sec at 55°C, and elongation for 1.5 min at 72°C. After cycling, relative quantitation of MGMT mRNA against an internal control, GAPDH, was possible by the following a ΔCT method. An amplification plot that had been the plot of fluorescence signal *vs*. cycle number was drawn. The parameter CT (threshold cycle) was defined as the fractional cycle number at which the fluorescence had passed the fixed thresholds. The difference (ΔCT) between the mean values in duplicated samples of MGMT and those of GAPDH was calculated by Microsoft Excel and the relative quantitation value (RQV) was expressed as $2^{-\Delta CT}$.

Statistical analysis

Statistical analyses were performed with SPSS statistical software, version 20.0 for Windows (SPSS IBM, IL, USA). Pearson chi-square test was performed to analyze the distribution of hypermethylation in test groups compared with control. Statistical significance was defined as $P < 0.05$.

Results

Patients and tumor characteristics

A total of 140 consecutive surgically resected FFPE tissue specimens comprising of 70 tumor tissues and 70 adjacent normal control regions of primary sporadic CRC were collected for analysis in the present study. Their ages ranged from 35 to 87 years (median, 66 years). Patients included 47 men (67.1%) and 23 women (32.9%). Of the 70 cases analyzed, tumor was located in the colon, rectum and rectosigmoid regions in 55.7%, 31.4% and 12.9% cases, respectively. Histological examination was done in all of the cases to evaluate tumor histotype. Details of each patient related to their demographic profile, clinical characteristics and methylation status of *MGMT* in tissues have been shown in table 2.

Table 1. Primer sequences used in methylation-specific PCR (Abdel Wahab *et al*., 2011).

Gene	Profile	Sequence
MGMT	Unmetylated	5'-TTTGTGTTTTGATGTTTGTAGGTTTTTGT-3' (sense)
		5'-AACTCCACACTCTTCCAAAAACAAAACA-3' (antisense)
	Metylated	5'-TTTCGACGTTCGTAGGTTTTCGC-3' (sense)
		5'-GCACTCTTCCGAAAACGAAACG-3' (antisense)

Methylation profile of MGMT

Methylation in *MGMT* promoter was detected in 32.9%of tumor tissues and 7.1% in adjacent normal control regions. Then aberrant methylation status significantly correlates with occurrence of CRC (p-value 0.000).

There is significant relation between tumor staging II with methylation status (p-value 0.308), but not significant relation between tumor differentiation (P value 0.442) and tumor location (P value 0.297) with methylation status. As it is shown in table 3, the correlation of methylation status with gender and age and is also not significant (p-values 0.186, 0.533, respectively).

Expression analysis of MGMT by qPCR

We performed a quantitative -PCR assay to analyze the mRNA expression of *MGMT* in FFPE tissue samples of colorectal cancer and normal adjacent tissue. We could detect *MGMT* mRNA expression in normal tissue than tumor is 8.7- fold. Therefore, *MGMT* expression was significantly reduced in colorectal cancer compared to normal adjacent tissue. As expected, expression of *MGMT* mRNA was clearly decreased in colorectal cancer stage II compared to the expression in normal adjacent tissue. The amount

of MGMT mRNA did not differ significantly between tumor grades.

Discussion

We know that colorectal cancer is one of the most important problems for human, and loss of MGMT expression is almost exclusively associated with methylation of CpG islands in the *MGMT* gene promoter region. MGMT protects normal cells from exogenous carcinogens, and inactivation of MGMT can induce DNA mutations and lead to disorders and cancers. We conducted this study to examine whether MGMT promoter aberrant methylation or reduction of its expression has any role in colorectal cancer risk. The incidence of aberrant methylation in *MGMT* promoter region in colorectal tumors, reported in some studies (Chan *et al*., 2002). It is suggesting that *MGMT* methylation is an early event in neoplastic pathway. Also, low level methylation of *MGMT* has been reported in normal colon mucosa in patients with a correspondingly *MGMT* methylated tumor, as well as individuals without colon cancer (Hawkins *et al*., 2005; Shen *et al*., 2005; Ye *et al*., 2006, Svrce *et al*., 2010; Worthley *et al*., 2010).

Table 2. Primer sequences used in qPCR (Tanaka *et al*., 2003; Caradec *et al*., 2010).

Gene	Sequence
MGMT	5′- CCTGGCTGAATGCCTATTTC -3′ (sense)
	5′- GATGAGGATGGGGACAGGATT-3′ (antisense)
GAPDH	5′-GCTCTCTCCTCCTGTTCG-3′ (sense)
	5′-ACGACCAAATCCGTTGACTC-3′ (antisense)

In our study 32.9% of cases had aberrant methylated in the promoter of MGMT that is moderate between other studies. Sinha *et al* (2013) have shown that the incidence of MGMT hypermethylation in Indian population is 47%. Menigatti *et al.* reported the degree of methylation in MGMT to be 18% (Menigatti *et al*., 2009). In turkey, the rate of methylation reported of MGMT in Pehlivan *et al* study was 35.3% (Pehlivan *et al*., 2010). Totally, MGMT promoter hypermehylation has been detected in 18–61% cases in CRC (Menigatti *et al*., 2009; , Abouzeid *et al* ., 2011; Shima *et al*., 2011; Shen *et al*., 2005; , Chen *et al*., 2009).

These findings suggest a role for *MGMT* methylation in sporadic colorectal cancer carcinogens. Therefore, it has been supposed that *MGMT* profile might be a useful marker for early detection and risk assessment in sporadic colorectal cancers.

In various studies, it was also revealed that *MGMT* methylation related with other cancers, such as, glioma and lung cancer (Vlassenbroeck *et al*., 2008; Gu *et al*., 2013).

In this study for first time, expression of MGMT in CRC was investigated by q-PCR, however, another study validated this method for investigation it in glioma (Vlassenbroeck *et al*., 2008). Previous studies on colorectal cancer have shown that MGMT methylation is associated with MGMT loss (Shen *et al*., 2005; Nagasaka *et al*., 2008; Whitehall *et al*., 2001). In agreement with these studies, the results of our current study have shown a significant correlation between aberrant methylation and reduced expression of *MGMT*, as *MGMT* methylated tumors showed reduced expression. The reduced expression of MGMT in some tumors lacking methylation suggested that not only the methylation but also genetic alterations are responsible. This other genetic alteration could possibility be mutation or polymorphism in the promoter or coding region of *MGMT* gene which could reduce the expression of MGMT mRNA in tumors lacking methylation. The illustration of the relationship between MGMT expression and *MGMT* gene methylation in primary tumors requires further studies on a large number of samples and this may certainly help us to better understand the role of methylation of tumor suppressor genes in carcinogenesis.

Some investigators have shown that

hypermethylation of the *MGMT* gene was not associated with advanced tumor stage, age, and gender (Shen *et al*., 2005; Abdel Wahab *et al*., 2011). However, in our study established that there is not relationship between age and gender with MGMT methylation but, is a significant inverse relationship between tumor stage with methylation.

Table 3. Demographic and clinical characteristics of 70 CRC tissues.

Patient characteristic	No. of samples (%)	MGMT methylatation status (tumor tissue)		P value Pearson Chi - Square
		No. of M (%)	No. of UM (%)	
Total	70 (100)	23(32.9%)	47(67.1%)	
Gender				
Male	47 (67.1)	13	34	0.186
Female	23 (32.9)	10	13	
Age				
≤50	28 (40)	8	20	0.533
50 <	42 (60)	13	27	
Tumor location				
Colon	39(55.7%)	11	28	0.297
Rectum	22(31.4%)	10	12	
Rectosigmoid	9(12.9%)	2	7	
Tumor Stage				
I	17(24.3%)	3	14	0.308
II	11(15.7%)	4	17	
III	37(52.9%)	13	24	
IV	5 (7.1%)	3	2	
Tumor grade#				
WD	17(24.3%)	3	14	0.442
MD	10(14.3%)	4	6	
PD	33(47.1%)	13	20	
U	10(14.3%)	3	7	

UM, unmethylated; M, methylated;

#WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated; U, undifferentiated.

p value <0.05 was taken as significant.

The present study showed that there was aberrant methylation in the adjoining tissues, too, and it suggests that normal tissues may contaminant with tumor tissue. This is the first study in the Iranian population in which *MGMT* gene has been analyzed at both epigenetic and expression level in CRC in relation to clinicopathological features and prognosis and confined to North East Iran. We need more data from the other parts of the country to validate our findings.

In conclusion, our study demonstrated that aberrant methylation *MGMT* can reduce its expression among tumor tissues in CRC, and may inversely contribute to tumor stage progression.

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