RESEARCH ARTICLE

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O6-Methyguanine-DNA Methyl Transferase (MGMT) Promoter Methylation in Serum DNA of Iranian Patients with Colorectal Cancer

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Abstract

Introduction: Colorectal cancer (CRC) is a leading cause of cancer deaths worldwide but current molecular targeted therapy is not providing major success in CRC treatment, so early detection by non-invasive methods continues to be vital. Aberrant methylation of CpG islands in promoter regions is associated with inactivation of various tumor suppressor genes. O6-methyguanine-DNA methyltransferase (MGMT) is a DNA repair enzyme that removes mutagenic and cytotoxic adducts from O6-guanine in DNA. Aberrant hypermethylation of the MGMT promoter has been associated with lack of mRNA expression, with concomitant loss of protein content and enzyme activity. **AIM:** Our aim was to determine whether MGMT promoter methylation might be detectable in circulating free DNA in the serum of CRC patients and normal individuals using a methylation specific (MSP) polymerase chain reaction (PCR) method. **Methods:** A total of 70 subjects were enrolled in the study. Of these, 30 patients who were diagnosed previously as untreated colon adenocarcinoma by a gastroenterologist and the other 40 were nearly age-matched individuals who had a normal colonoscopic evaluation (except for hemorrhoids or fissures) and normal pathologic reports. After bisulphite modification of DNA, serum samples were examined for MGMT promoter methylation using MSP. **Results:** Ninety percent of CRC patients had MGMT promoter hypermethylation as compared to no methylation in normal subjects' serum. Most of the cancers were stage Π and moderately differentiated adenocarcinomas; nearly 60% were found in the left colon. No statistically significant correlation was found between the promoter methylation status and gender and age. **Discussion and Conclusions:** MGMT hypermethylation can be detected in free circulating DNA in serum of CRC patients and can be used "as a clinical biomarker" for early diagnosis and prognostic assessment of the disease. Our data confirm previous studies indicating utility for free circulating DNA as a serum biomarker for early detection, diagnosis and monitoring of CRC patients.

Keywords: MGMT- colorectal cancer- promoter methylation- Iranian patients

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Introduction

Colorectal cancer (CRC) is one of the most common cancer, affecting 1.23 million individuals per year included 9.7% of overall cancers (Dolatkhah et al., 2015 a; Stigliano, 2014). Despite advances in surgery, chemotherapy and screening for the diagnosis and treatment, the prognosis of advanced CRC is still poor and the incidence of CRC death is high (Raskov, 2014). In developed countries, CRC is more prevalent in older age groups and it is the third leading cause of cancer death (Haggar and Boushey, 2009; Siegel et al., 2014; Stigliano, 2014). As compared with Western population, its overall incidence is low In Iran (Dolatkhah et al., 2015b; Haghdoost et al.), but it is increasing in younger age groups (Nelson et al., 1998; Wu et al., 2004; Tezcan, 2016; Malekzadeh et al., 2009).

The risk of developing CRC is impacted by both environmental and genetic factors (Azadeh et al., 2008; Li and Lai, 2009; Malekzadeh et al., 2009; Nelson et al., 1998; Tezcan, 2016; Wu et al., 2004).

Colorectal cancers has one of the three following patterns: sporadic (70%), familial without documented inherited genetic pattern (25%) and inherited by genetic pattern (less than 10%). The hereditary CRC group is subdivided as familial adenomatous polyposis (FAP) and MUTYH-associated polyposis (MAP) based on the presence of polyp and hereditary nonpolyposis CRC (HNPCC; Lynch syndrome) where no polyps have

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been found but there is a high risk for developing CRC (Giardiello et al., 2014).

Molecular multistep pathogenesis of CRC is complex which not only may be due to the genetic alteration of primary nucleotide sequence of a gene, but also could be a consequence of association between genetic and epigenetic alterations (Armaghany et al., 2012; Ballestar and Esteller, 2008; Lao and Grady, 2011; Okugawa et al., 2015). Chromosomal instability (CIN) is the most common pathway associated with genomic instability in CRC (Giam and Rancati, 2015). Moreover, some sporadic CRC arise as a consequence of Microsatellite instability (MSI) but they typically are due to aberrant methylation of MLH1 promoter and they are not the result of germline alteration as seen in lynch tumors. Aberrant DNA methylation, abnormal histone modification, altered noncoding RNAs (including microRNA) are the main epigenetic alteration seen in cancers (Chan et al., 2002; van Engeland et al., 2011; Grady and Carethers, 2008; Mokarram et al., 2013; Rashid et al., 2001). The epigenetic alterations may also be seen in precancerous conditions such as polyps and inflammatory bowel diseases (Dhir et al., 2008; Mokarram et al., 2015a, 2015b; Rashid et al., 2001).

In human, aberrant hypermethylation of certain CpG sites in promoter region of tumor suppressor genes can lead to gene silencing and loss of function, leading to cancer initiation and progression in CPG island methylator phenotype (CIMP) cancers. This common aberrant molecular alteration have been seen in colorectal cancers (Esteller et al., 1999; Grady and Carethers, 2008; Lee et al., 2009; Mansour, 2014; Qi, 2005). Based on literature review, the promoter hypermethylation occur in early stages of disease and examining the gene status in serum is helpful for early detection of malignant status (Ma et al., 2010).

Epigenetic alterations, have an important role in CRC cancer initiation and progression and the associated level in tissue, plasma and stool (but not normal mucosa) of patients, suggesting as a potential biomarkers for CRC detection and prediction of the progression or drug responsiveness (Lao and Grady, 2011)

Biomarkers have many potential applications in Oncology, including risk assessment, screening, differential diagnosis, determination of prognosis, prediction of response to treatment, and monitoring the progression of the disease. So, prior to incorporation of biomarkers into routine clinical care, their critical roles mandate rigorous evaluation including analytical and clinical validation, and assessment of clinical significance (Henry and Hayes, 2012a). Detection of methylated genes (as biomarkers) in various body fluids such as blood, urine, fecal and tissue samples are attracting the interest of investigators (Yi et al., 2012).

In this regard, hypermethylation of CDKN2A, a tumor suppressor gene, APC, Secreted frizzled-related protein 4 (sFRP4), Secreted frizzled-related protein 1 (sFRP1), Beta-1, 4-Galactosyl transferase 1(B4GALT1), Tissue factor pathway inhibitor-2 (TFPI-2), mutL homolog 1 or colon cancer, nonpolyposis type 2 (hMLH1), FRP4, FRP5, GATA-4 and TMEM25 has been reported in several studies and all are potent biomarker candidates (Nakayama et al., 2002; Syed Sameer and Nissar, 2016; Wang, 2014; Yi et al., 2012). Genetic alteration in O6-methylguanine-DNA methyl transferase (MGMT), deteriorate the ability of the MGMT protein to remove alkylating group from the O6 -position of guanine and consequently increase the rate and risk of malignancy. In addition, the association of aberrant hypermethylation of MGMT promoter with lack of mRNA expression, and loss of protein and enzyme activity has been reported (Lee et al., 2009; Mokarram et al., 2015a; Naini et al., 2016a) and It has to be taken into consideration further in future investigations.

In previous studies, our data showed that MGMT-B methylation not MGMT-A is associated with KRAS mutation in tissues of CRC patients (Mokarram et al., 2013). Furthermore, we reported novel hypermethylation of MGMT-B in patients with polyp and inflammatory bowel disease (Mokarram et al., 2015b; Naini et al., 2016b).

The present study is designed to evaluate the detection rate of aberrant MGMT-B in serum of patients with colon cancer.

Mareials and Methods

Patient Selection

Seventy patients were enrolled in this study. Out of that, 30 patients were diagnosed with colorectal tumor and have not been treated and 40 cases of healthy individuals have been enrolled as controls. The selection of patients was based on colonoscopy procedure and histopathology reports. Our sample population only confined to sporadic CRC, hence patients with family history of colorectal cancer were excluded from this study. Prior to the study, all patients and controls have signed informed consent form, for the collection of serum specimens. The subsets of patients referred to GI wards of Shiraz Teaching Hospitals were chosen between 2013 and 2014. The study protocol was approved by the Institutional Review Board of Shiraz University of Medical Sciences.

Sample collection

All 70 serum samples were obtained during colonoscopy examination. Five milliliter peripheral venous blood samples were withdrawn from all participants by venipuncture. Samples were immediately centrifuged at 3,000 g for 10 minutes to separate the serum and stored at -80°C, until further use.

DNA extraction

Free circulating DNA (fcDNA) was extracted from 500 µl serum samples using EZ DNA Methylation Kit (Zymo Research, Orange, CA, USA), according to the manufacturer's instructions and stored at -80 ^{o}C, until further use.

Bisulfite modification

The modification of serum fcDNA was performed using the EpiTect Bisulfite Kit (Qiagen), converting only unmethylated cytosines to uracils, according to the manufacturer's instructions.

Table 1. Primer Sequences and Specific Annealing Temperature

primers	Sequence (5′-3′)	Annealing Temperature	Product size
MF	GGTCGTTTGTACGTTCGC	59	127 bp
MR	TAACCCTTCGACCGATACAA	59	127 bp
UF	GTAGGTTGTTTGTATGTTTGT	59	127 bp
UR	TAACCCTTCAACCAATACAAACC	59	127 bp

Methylation Specific PCR (MSP)

In brief, this technique uses bisulfate modification to convert unmethylated, but not methylated, cytosine to uracil. MSP utilizes this difference to amplify specifically either methylated or unmethylated DNA. Locus specific PCR primers for MGMT-B promoter region was designed for methylation specific PCR (MSP) amplification. The primer sequences, annealing temperature specific to each primer, and size of PCR products were described in Table 1. The hot-start Taq polymerase reactions were performed in a 50 µL reaction containing 25 pmol of sense and antisense primers, 0.2 mM/L dNTPs, and 80 µg bisulfite-modified DNA in 1× PCR buffer . The reaction mixture was denatured at 95°C for five minutes, followed by addition of 1.5 U Taq polymerase; then 40 cycles of amplification was performed, each consisting of 30s denaturation at 95°C, proper annealing temperature for MGMT (Table 1) and 30s polymerization at 72°C, followed by a single 10-minute extension at 72°C. The universal methylated DNA (chemicon) was used as positive control for methylated alleles of MGMT-B and DNA from normal lymphocytes was used as the negative control. Then, 10 µL of amplified PCR products were mixed with 5 µL of loading dye and electrophoresis was performed on 2.5% agarose gel containing gel red using TBE buffer and visualized under UV illumination (Naini et al., 2016a).

Statistical Analysis

Statistical analysis of promoter methylation of MGMT gene was performed with either the Chi-square or the Fisher's exact tests. The level of significance was $p < 0.05$. All statistical data were analyzed by SPSS software, version16 (SPSS Inc., Chicago, IL).

Results

In this study, the methylation status of MGMT promoter was analyzed using serum from 30 CRC patients and compared with 40 normal controls. The pathological and clinical characteristics of the patients are presented in Table 2.

Table 2. Characteristics of Patients with Colorectal Cancer in Relation to the Methylation Status of *MGMT* Promoters

Characteristics	Methylated *MGMT (%)*	Unmethylated *MGMT(%)*	p value
Gender			
Female	12 (40%)	2 (6.7%)	0.46
Male	15 (50%)	1 (3.3%)	
Location			
Right	9 (30%)	0 (0%)	0.23
Left	18 (60%)	3 (10%)	
Stage			
I	4 (13.3%)	2 (6.7%)	0.1
II	17 (56.7%)	0 (0%)	
III	5 (16.7%)	1 (3.3%)	
IV	1 (3.3%)	0 (0%)	
Differentiation			
Poor	2 (6.7%)	0 (0%)	0.87
Moderate	18 (60%)	2 (6.7%)	
Well	7 (23.3%)	1 (3.3%)	

As shown in Table 2, most CRC patients had left colon tumors (60%). All of tumors were adenocarcinoma, moderately differentiated and the stage of colorectal cancer was mostly stage II. In total, in 90% (n=27) of the patients methylation of MGMT promoters was detected.

The MSP was performed on all serum samples and representative of methylation profile was shown in Figure 1. No statically significant correlation was found between genders, age with the promoter methylation status. However, there is a statistically significant difference between patients and control group according to methylation status ($p<0.0001$). The prevalence of MGMT methylation was high, in 90% of CRC patients, whereas there is no methylation in normal controls.

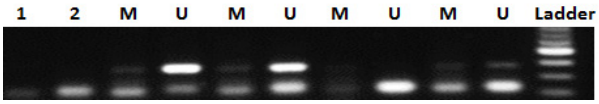

Figure1. Representative Example of MGMT Methylation Assay by MSP. The presence of a visible PCR product in lanes marked as U, indicates the presence of unmethylated genes; the presence of a product in lanes marked M, indicates the presence of methylated genes. Lane 1, 2 indicates NTC samples without template DNA. Universal methylated DNA (UMD) and unmethylated lymphocytes (lymphocytes) DNA was used as positive and negative controls.

Discussion

Based on significant mortality and morbidity in CRC patients, especially with delayed diagnosis, early detection of tumor or predisposing conditions such as genetic or epigenetic susceptibilities is vital (Haggar and Boushey, 2009). That is also valid for patients who are more prone to CRC such as inflammatory bowel diseases or the people with prolong contact with hazardous environmental pollutions (Dhir et al., 2008). Although colonoscopy is a gold standard procedure of choice for early detection of

CRCs or polyps but it is an invasive, time-consuming and expensive procedure, which requires patients' tolerance. Prevention or early detection using a non-invasive method has been the main goal of researchers. Documented role of epigenetic alteration in cancer development have attracted attention of researchers in this field. The comparative studies between normal and tumor cell profile, preclinical studies using xenograft tumors and attempt to find biomarkers are the main area of research in CRC (Henry and Hayes, 2012b).

Biomarkers have also the potential to differentiate between diagnostic and treatment tests leading to selection of proper chemotherapeutic drugs for specific patient. Biomarkers are also seen as the key to personalized medicine as there are attempts to personalize chemotherapy based on presence or absence of specific biomarkers. Goal of future research is to identify those biomarkers that could allow a non-invasive and cost-effective diagnosis, as well as to recognize the best prognostic panel and define the predictive biomarkers for available treatments (Lech, 2016).

MGMT silencing due to aberrant methylation, but not the documented MGMT genetic defects, is detected in various type of cancers and the detection in free circulating DNA or other samples such as stool is also possible. In 2005 Shen et al., (2005) detected MGMT promoter methylation both in tumor and in adjacent mucosa. The result was consistent with the findings of Mokarram et al., (2015a) stated the predictive significance of circulating methylated DNA in serum of melanoma patients receiving bio-chemotherapy. In 2015, Wang (2014) and Kadiyska and Nossikoff, (2015) reported detection of aberrant MGMT methylation in serum and stool of CRC patients as a screening method, suggesting to be used as a prognostic factor in CRC.

Hibi et al., (2011) also detected MGMT methylation in gastric and colorectal cancers (Kadiyska and Nossikoff, 2015). In our previous study, we have shown that MGMT-B methylation or silencing but not MGMT-A was significantly associated with KRAS gene mutation in colorectal cancers (Mokarram et al., 2013). Variable methylation profiles were also detected in precancerous states; in 2015, Psofaki et al., (2010) showed the methylation profiling in both in tubulovillous/villous adenomas as well as colorectal cancers. In another study, we detected hypermethylation of MGMT-B and SFRP2 in patients with inflammatory bowel diseases, which has a potential to be used for early detection of high risk IBD patients and prevention of colorectal cancer (Mokarram et al., 2015b). The non-invasive and sensitive detection of aberrant SFRP2 and MGMT-B methylation in Iranian patients having polyps in colon, is supporting the significance of procedure for detection of high risk patients, in order to be prioritized for colonoscopic evaluation (Naini et al., 2016a). Current study is designed to examine the MGMT gene promoter methylation status in serum of CRC patients. This also may be used as a potential tool in clinical settings. The high frequency of detecting abnormal methylation in MGMT in this study (90%), not detected in age, sex-matched healthy population, is supporting its association with colorectal cancer. In addition, the simultaneous detection of methylation in both tissue and serum reveals a highlighted role as a biomarker for CRC detection by a non-invasive method. In line with the same results, Hang et al. detected abnormal methylation of MGMT in CRC patients but not in normal controls (Huang et al., 2007). With respect to sensitivity, there are some conflicting results; Detection rate of aberrant circulating MGMT was 39%, 38% and 48.1% in Lee et al., (2009); Huang et al., (2007) and Shima et al., (2011) studies, respectively. Although stool DNA test are also investigated for CRC detection as an easy and non-invasive method, but due to relatively low positive predictive value, poor patient compliance and less significance, evaluation of MGMT promoter region was only performed in serum samples (Kadiyska and Nossikoff, 2015; Mazilu, 2014). In this study, due to limited storage facility, stool samples have not been tested.

This study was performed on 70 cases (30 patients and 40 controls) with relatively small size, so larger sample size is required in order to convey more information and find biomarkers that are more reliable and valid.

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