Original Article

Hypermethylation of *MGMT* Gene Promoter in Peripheral Blood Mononuclear Cells as a Noninvasive Biomarker for Colorectal Cancer Diagnosis

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Abstract

Background: Early colorectal cancer (CRC) diagnosis can drastically reduce CRC-related morbidity and mortality. In this regard, increasing attention is now being directed to DNA-based tests, especially the evaluation of methylation levels, to prioritize high-risk suspected persons for colonoscopy examination. Therefore, we aimed to assess the accuracy of *MGMT* gene promoter methylation levels in peripheral blood mononuclear cells (PBMCs) for distinguishing CRC patients from healthy people.

Materials and Methods: For this study, a total of seventy individuals with CRC and 75 healthy individuals from Iran were included. The methylation level of *MGMT* in the DNA isolated from PBMCs was evaluated using the methylation quantification endonuclease-resistant DNA technique. To assess the diagnostic capability of the *MGMT* promoter methylation level, a receiver operating characteristic (ROC) curve was generated.

Results: The mean promoter methylation level of *MGMT* in the CRC and control groups was, respectively, 27.83 ± 22.80 vs. 12.36 ± 14.48. The average percentage of methylation of the *MGMT* promoter between the CRC and control groups was significantly different ($P < 0.001$). Also, the MGMT promoter was more hypermethylated in female patients than in males. ROC analyses indicated that the diagnostic power of the *MGMT* promoter methylation level for CRC was 0.754, with a sensitivity of 81.43% and a specificity of 75.71%, indicating a good biomarker for CRC diagnosis.

Conclusion: Methylation evaluation of *MGMT* in PBMCs could be utilized as a diagnostic biomarker with high accuracy for prioritizing suspected CRC patients before colonoscopy.

Keywords: Biomarker, colorectal cancer, methylation, *MGMT*

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Submitted: 13-Jun-2023; **Revised:** 10-Aug-2023; **Accepted:** 13-Aug-2023; **Published:** 29-Nov-2023

Introduction

According to the last update of GLOBOCAN, colorectal cancer (CRC) was the second leading cause of cancer-related mortality in 2020 in both men and women worldwide.[1] The incidence of CRC in Iran has been dramatically on the rise in the last decade, especially among young people.[2,3] Evidently, early diagnosis is the most important aspect to reduce CRC-related death. In this regard, colonoscopy, as the current gold standard method for the detection of CRC, has

Access this article online

Quick Response Code:

Website: www.advbiores.net

DOI: 10.4103/abr.abr_206_23

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How to cite this article: Azhdari S, Khodabandehloo F, Ehtesham N, Mazhari SA, Behroozi J, Siri G. Hypermethylation of *MGMT* gene promoter in peripheral blood mononuclear cells as a noninvasive biomarker for colorectal cancer diagnosis. Adv Biomed Res 2023;12:256.

achieved a successful gain, but its procedure is accompanied by inconvenience.[4] Hence, researchers are striving to find tests with more comfortability and higher accuracy to prioritize high-risk suspected individuals for colonoscopy evaluation. In this context, two available non-invasive screening methods, that is, Fecal Occult Blood Test and Fecal Immunological Test, have low sensitivity and specificity, but DNA-based tests have shown promising findings.[5]

Epigenetics is defined as the regulation of gene expression without alteration in the underlying DNA sequence by mechanisms including DNA methylation, histone modifications, and non-coding RNAs such as microRNA.[6-8] It has been evidenced that reduced activity of many DNA repair genes, including O^6 methyl-guanine methyltransferase (*MGMT*), by somatic epigenetic silencing leads to increased DNA damage, which is critically involved in the pathogenesis of many cancers.[9] In previous research, numerous studies have documented that the somatic epigenetic inactivation of *MGMT* through hypermethylation takes place during the early stages of CRC. This process plays a crucial role in the step-by-step progression from normal adenoma to carcinoma, occurring before the development and advancement of CRC.[10,11] Therefore, it has been suggested that *MGMT* hypermethylation functions as a "field defect" in cases of sporadic colon cancer. This means that there is a molecularly abnormal area of tissue that precedes and increases the susceptibility to cancer development.[12] Seemingly, this constitutional epimutation is partially recognized for its connection to the buildup of mutations in the *KRAS* and *TP53* genes. This association is attributed to the primary role of MGMT in preventing G:A transitions.[13-15] Consequently, there has been extensive research on *MGMT* methylation status as a diagnostic biomarker for CRC diagnosis. This investigation has primarily focused on stool and serum samples rather than blood cells.[16,17] The utilization of peripheral blood mononuclear cells (PBMCs) as biomarkers, particularly in various cancer types, has garnered significant interest. This is due to their ability to indirectly replicate the epigenetic patterns found in affected tissues.[18] In this study, for the first time, we evaluated the methylation pattern of the *MGMT* promoter in PBMCs of CRC patients compared with healthy controls to determine whether differential methylation of this gene in PBMCs could be used as a diagnostic biomarker of CRC.

Materials and Methods

Sample collection

A total of 145 participants were included in this study, with blood samples collected simultaneously. Among them, 70 cases were identified as CRC patients through colonoscopy, and diagnostic information such as pathological confirmation and staging of CRC was obtained for each patient. The remaining 75 individuals had negative colonoscopy results and no personal or familial history of cancer, serving as the control group. Demographic characteristics, including age, sex, smoking status, and body mass index (BMI, calculated as weight [kg] divided by the square of height [m]), were recorded using a structured questionnaire. Ethical approval was obtained from the research ethics committee with approval number IR.AJAUMS.REC.1400.189, and informed consent was obtained from each volunteer after providing them with comprehensive information about the study. A volume of 2.5 ml of peripheral blood was collected from each of the 145 participants using EDTA tubes and stored at -20°C until further processing.

Blood mononuclear cell preparation and DNA isolation

Following the established standard procedure,[19] PBMCs were isolated using density gradient centrifugation with Ficoll-Hypaque (Sigma, St. Louis, MO, USA) from the collected blood samples. Genomic DNA was then extracted from the PBMCs using the Prime Prep Genomic DNA Isolation Kit (GeNetBio, Korea). The quality and quantity of the extracted DNA were assessed through agarose gel electrophoresis and a NanoDrop Spectrophotometer, measuring the absorbance ratio at 260 nm.

MGMT methylation analysis

Quantitative methylation analysis was performed using the methylation quantification endonuclease-resistant DNA (MethyQESD) method. In this technique, methylation-sensitive digestion and real-time polymerase chain reaction (RT-PCR) are combined. This method was described by Bettstetter *et al*.[20] for the first time. Digestion was performed by the methylation-sensitive endonuclease Hin6I and the methylation-insensitive enzymes XbaI and DraI that digest total DNA except for our target promoter sequences in two different batches. The sequences of primers were as follows: *MGMT* forward: 5'-CCCGGATATGCTGGGACAG-3'; *MGMT* reverse: 5'–CCCAGA CACTCACCAAGTCG-3'. The cycling profile started with 10 minutes of initial denaturation at 95°C, then 45 cycles of amplification, including 95°C for 15 seconds, 60°C for 20 seconds, and finally 72°C for 30 seconds. The digestion protocol and components of the PCR reaction mixture for *MGMT* RT-PCR were documented in detail in a study by Duppel *et al*.[21]

Statistical analysis

The statistical tests to evaluate the methylation percentage of the *MGMT* promoter sequence in the CRC and control groups were performed by SPSS version 25 (Armonk, NY: IBM Corp.). *P* values were calculated by independent t-tests and Chi-square tests. The diagnostic performance of the *MGMT* methylation was assessed by the receiver operating characteristic (ROC) curves. The sensitivity (true CRC/true CRC+ false CRC-free), specificity (true CRC-free/true CRC-free + false CRC), and respective areas under the curve (AUC) were determined to identify the best cutoff values for the percentage of DNA methylation that can discriminate CRC patients from healthy people. The significance level was set at $P < 0.05$.

Results

Demographic and laboratory characteristics

In this study comparing cases and controls, a total of 70 patients with CRC (39 males and 31 females, with an average age of 56.02 ± 11.46) and 75 healthy individuals (34 males and 41 females, with an average age of 54.70 ± 9.39) were analyzed. The characteristics of the patients and healthy individuals are displayed in Table 1. Notably, there were no significant differences between the two groups in terms of sex, age, BMI, or smoking, indicating successful matching. Among the 70 patients, 20 were in stage I, 26 in stage II, 15 in stage III, and 9 in stage.

MGMT methylation analysis

The average level of promoter methylation for MGMT in the CRC group was 27.83 ± 22.80, while in the control group, it was 12.36 ± 14.48. The percentage of methylation in the MGMT promoter was significantly different between the CRC and control groups ($P < 0.001$). Figure 1 provides a comparison of the promoter methylation levels of *MGMT* between CRC patients and the healthy control group. The methylation level showed a significant increase with stage [Table 2; $P < 0.001$). Interestingly, the average percentage of methylation for MGMT was relatively similar between the two groups of CRC patients, those aged ≤54 and those aged >54 [Table 3; $P = 0.196$]. However, female patients had significantly higher levels of MGMT hypermethylation compared to male patients [Table 3; $P < 0.001$]. On the other hand, there was no statistical difference in the methylation levels between smokers (28.84%) and non-smokers (27.26%) in the patient group (*P*: 0.338).

Table 1: Characteristics of CRC patients and healthy controls in this study

Variable	Case (*n*: 70)	Control (*n*: 75)	*P*
Sex			
Male	39 (55.7%)	34 (45.3%)	0.246
Female	31 (44.3%)	41 (54.7%)	
Age (mean±SD)	56.02±11.46	54.70±9.39	0.447
BMI (mean±SD)	24.80±3.89	25.09±3.25	0.624
Smoker	23 (32.9%)	17 (22.7%)	
Non-smoker	47 (67.1%)	58 (77.3%)	0.196
Stage			
I	20 (28.57%)	--	
II	26 (37.14%)	--	
III	15 (21.43%)	--	
IV	9 (12.86%)	--	

BMI: Body mass index; SD: Standard deviation

Furthermore, the ROC analyses indicated that the *MGMT* promoter methylation level had a diagnostic power of 0.754 for CRC [Figure 2]. The optimal cutoff point for distinguishing CRC patients from controls based on the *MGMT* promoter methylation level was determined to be 10.36%, with a sensitivity of 81.43% and a specificity of 75.71% [Table 2].

Discussion

In this study, *MGMT* was found to be substantially hypermethylated in the PBMCs of CRC patients compared with healthy people. In addition, ROC curve analysis demonstrated that the methylation status of MGMT has good sensitivity and specificity for discriminating between CRC patients and disease-free individuals. Regarding the fact that methylation of *MGMT* during CRC development occurs very early,[22] we contend that evaluation of *MGMT* methylation level in PBMCs is a well-suited preliminary method for the timely detection of precancerous lesions by colonoscopy. At present, the FDA has approved two methylation-based diagnostic biomarkers for CRC, namely *SEPT9* and the combination of *NDRG4* and *BMP3*.[16] Considering the findings of our study and current publications about *MGMT* methylation as a diagnostic biomarker of CRC,[16] it seems that methylation assessment of this gene should be considered for future FDA validation.

Our analysis indicated that age does not affect the methylation pattern of *MGMT*, but the promoter region of this gene is more hypermethylated in female patients than male patients.

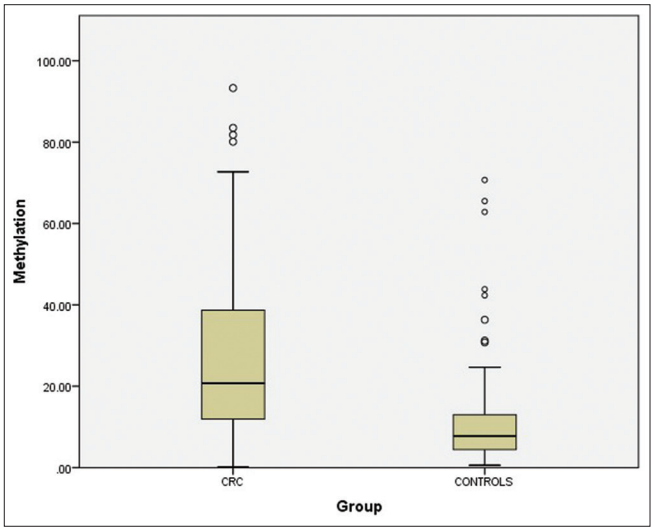

Figure 1: Comparison of MGMT promoter methylation levels between patients with CRC and healthy controls. $P < 0.001$*

Table 2: The percentages of methylation in the MGMT gene in case and control groups and diagnostic value

Group	AM	*P*	Cutoff	Sensitivity	Specificity	AM in I	AM in II	AM in III	AM in IV	*P*
MGMT										
Case (*n*: 70)	27.83±22.80	<0.001*	10.36%	81.43%	75.71%	17.23±10.67	22.94±18.41	40.67±31.03	44.10±22.49	<0.001*
Control (*n*: 70)	12.36±14.48					_	_	_	_	_

*$P<0.05$; AM: Average methylation; I: stage I; II: stage II; III: stage III; IV: stage IV

Table 3: The percentages of methylation in the MGMT gene in CRC patients in terms of sex and age

	Average methylation	*P*
Gender		
Male (*n*=39)	18.0706±14.96889	<0.001*
Female (*n*=31)	40.1154±25.15969	
Age		
≤54 (*n*=32)	31.9863±26.98818	0.196
>54 (*n*=38)	24.5322±18.55846	

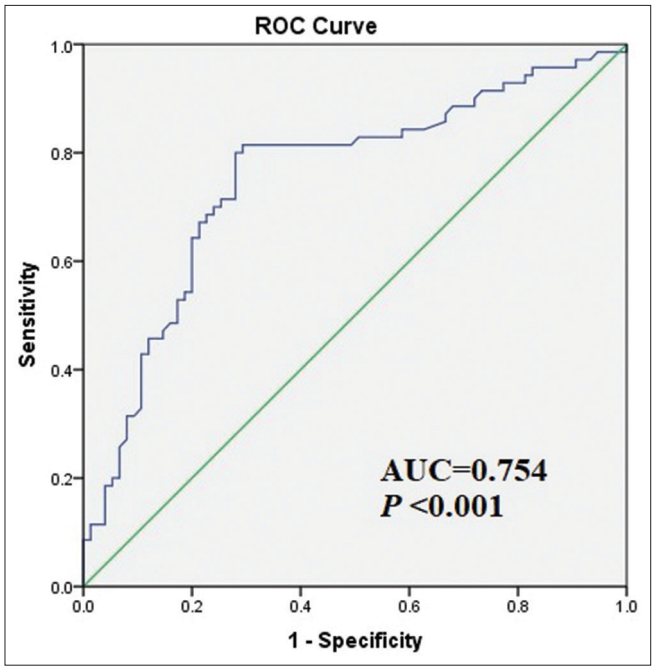

Figure 2: The receiver operating characteristic (ROC) curves of *MGMT* promoter methylation level in patients with CRC compared with healthy controls

In this context, previous studies reported inconsistent findings. Similar to our study, Zheng *et al*.[11] uncovered that *MGMT* hypermethylation was considerably higher in female patients than in male patients. In contrast, in two different works, Alizade Naini *et al*. showed that neither sex nor age were associated with the promoter methylation status of *MGMT*.[17,23] In addition, they reported no difference in average methylation of *MGMT* between different stages,[17] while we observed that methylation rises with the increase in stages, which reflects the diagnostic value of this biomarker even in determining the stage of disease in affected patients. In this context, Farzanehfar and colleagues revealed no difference in *MGMT* methylation in CRC patients in terms of age, sex, or tumor stage.[24] In another study in the Iranian population, the methylation level was independent of age and gender but increased with higher stages.[25] Lastly, in contrast to our results, a meta-analysis revealed that the frequency of *MGMT* methylation in male CRC patients is higher than that in female patients.[26]

Our study has some strengths that must be considered. Considering the effects of lifestyle-related methylation alterations such as smoking[27] and BMI,[28] patients and controls in our study were comparable in these indices. Also, the chosen technique for methylation assessment has superiority over other methylation profiling methods because the process of MethyQESD is devoid of bisulfite treatment of extracted DNA, preventing DNA degradation.[20] We demonstrated the accuracy, reliability, and high quality of MethyQESD for methylation analysis in the PBMCs of CRC patients in agreement with previous studies.[29-32] In this regard, Bagheri *et al*.[29] revealed that the methylation status of *TFPI2* and *NDRG4* genes in PBMCs has high sensitivity and specificity for the detection of CRC, and the combination of these genes provides sufficiently higher diagnostic power (AUC = 0.961), suggesting a promising noninvasive CRC screening approach.

Finally, we opted to use PBMCs as the specimen for investigation. This choice was made due to the ease and non-invasiveness of DNA methylation assays conducted on both stool and blood samples, including PBMCs. However, previous studies have demonstrated that stool DNA tests have a low positive predictive value and less significance. Additionally, patient compliance with stool DNA tests is low.[33,34]

Although there are several advantages to consider, it is important to acknowledge that there are also limitations that could affect the reliability of our results. One limitation is the potential impact of single nucleotide polymorphisms (SNPs) known as methylation quantitative trait loci (meQTL) on the regulation of DNA methylation machinery and its influence on methylation patterns.[35] It would be better if the association between meQTL of *MGMT* and its methylation pattern was assessed. Secondly, we did not assess the methylation status of cancerous colorectal tissues to see whether there is a correlation between the methylation level of PBMCs and the corresponding tissue.

Collectively, despite our promising findings, before incorporating the evaluation of *MGMT* methylation level in PBMC as a diagnostic test in clinical settings, validation in independent studies with a larger sample size is needed. Likewise, although a meta-analysis indicated that *MGMT* methylation is not appreciably associated with CRC prognosis,[26] we recommend that the methylation level of *MGMT* in PBMCs be investigated for other purposes such as measuring disease recurrence, tumor burden, and prognosis.

Conclusion

Altogether, we propose that the methylation assessment of *MGMT* in PBMCs could be employed as a diagnostic biomarker with high accuracy for prioritizing suspected CRC patients before colonoscopy. However, because of more hypermethylation *MGMT* in female patients, the results must be adjusted in terms of sex.

Ethics approval and consent to participate

This study was approved by the ethics committee of the AJA University of Medical Sciences.

Informed consent was obtained from all subjects. Also, all authors agree to publish this manuscript in your valuable journal.

Acknowledgment

We thank all the patients who participated in this study. Also, we would like to appreciate the financial support provided by AJA University of Medical Sciences.

Financial support and sponsorship

This study was financially supported by the AJA University of Medical Sciences.

Conflicts of interest

There are no conflicts of interest.

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