Original Article
Running Title: *MLH1* Methylation in PBMCs as a Biomarker for CRC
Received: May 07, 2024; Accepted: August 26, 2024

MLH1 Methylation-Based Testing in Peripheral Blood Mononuclear Cells is a Promising Biomarker for Colorectal Cancer Diagnosis and Prognosis

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Abstract

Background: Recent evidence has shown that peripheral blood mononuclear cells (PBMCs) can reflect the epigenetic profile of tissues they interact with, such as malignant cells. The hypermethylation of MLH1 promoter is a well-defined epigenetic alteration in the development of colorectal cancer (CRC). This is the first study aimed to assess the diagnostic and prognostic values of the methylation level of *MLH1* promoter in PBMCs of patients with CRC.
Method: In this case-control study, the methylation level at the promoter region of MLH1 was quantitatively analyzed in 60 CRC patients and 60 non-cancerous study participants via methylation-quantification of endonuclease-resistant DNA (MethyQESD). The receiver operating characteristic (ROC) curves were constructed and the areas under the curve were calculated to determine the diagnostic significance of *MLH1* gene methylation.
Results: Our data showed a significant increase in methylation of *MLH1* in CRC patients compared with healthy participants ($P < 0.001$). Moreover, the specificity of *MLH1* hypermethylation for precise diagnosis of healthy participants was 75% and the its sensitivity for CRC diagnosis was 76.7%. With ROC curve analyses, we found that *MLH1* promoter methylation holds a likelihood of 76.8% for distinguishing between CRC patients and healthy individuals ($P < 0.001$). Besides, *MLH1* methylation levels was significantly increased in CRC patients with higher tumor stages, suggesting a probable correlation between an increased percentage of methylation and tumor progression ($P < 0.001$). However, no statistically significant association was found between methylation status of *MLH1* and microsatellite instability ($P > 0.05$).

Conclusion: Our results propose that *MLH1* methylation status in PBMCs can be used as a promising diagnostic and prognostic biomarker and reliable factor for CRC screening.
Keywords: *MLH1*, Colorectal neoplasms, Biomarkers, Diagnosis

Introduction

Colorectal cancer (CRC) is the third most common cancer and the second leading cause of cancer-related deaths worldwide. The majority of CRCs originate as polyps, and it typically takes 10 years for a small polyp to progress into cancer.[1] Studies have shown that the 5-year survival rate for CRC is around 90%, when discovered in early stages.[2] Colonoscopy is widely considered as the gold standard method for CRC screening; however, it is known as an invasive procedure with a non-negligible risk of major complications.[3,4] Furthermore, tissue samples (biopsies) need to be collected during the procedure for histopathological assessment to determine specific clinical indications such as tumor stages and microsatellite instability (MSI) status.[5] Therefore, researchers are currently focus on developing more comfortable and accurate tests for the early diagnosis of CRC in high-risk individuals and predicting their prognosis.

In recent years, studies on blood -based biomarkers as a non-invasive and convenient approach for the early diagnosis and prognosis of CRC have drawn extensive attention. These biomarkers are based on circulating tumor DNA (ctDNA) and/or RNAs, capable of detecting tumor-associated DNA/RNA changes, including gene mutations, DNA methylation, and non-coding RNAs (ncRNAs) such as long non-coding RNAs (lncRNAs) and microRNAs (miRNAs).[6-8] However, the use of circulating tumor nucleic acids (ctNAs) is challenging due to their low quantity, high degree of fragmentation, and large amount of nonspecific background DNA, which can lead to low sensitivity and specificity.[9,10] Recently, researchers have been investigating blood cells such as leukocytes and peripheral blood mononuclear cells (PBMCs), as a new source of epigenetic biomarkers, particularly for cancer implications. Emerging evidence suggests that PBMCs can reflect the epigenetic profile of tissues they come into contact with, including malignant cells. The change in the epigenetic profile of PBMCs is thought to be mediated by cancer cell-derived exosomes, which contain miRNAs, lncRNAs, and other regulatory molecules.[11,12] Previous studies have shown that the methylation profile of the whole genome and specific genes, such as *NDRG4*, *TFPI2*,[13] *TUSC3*,[14] *MMP9*,[15] *ITGA4*,[16] *MGMT*,[17] and *RUNX3*,[18] is subjected to alterations in PBMCs of patients with CRC.

MLH1 is the most commonly dysregulated DNA mismatch repair (MMR) gene in CRC. The hypermethylation of the *MLH1* promoter is a well-known epigenetic alteration that is commonly observed in the development and progression of CRC.[19] *MLH1* promoter methylation is frequently detected in sporadic microsatellite unstable CRC tumors. Also, it is correlated with certain clinical characteristics.[20,21]

In this study, we aimed to assess the methylation levels of *MLH1* in the PBMCs of patients with CRC and healthy controls. This is the first study to investigate whether differential methylation of *MLH1* in PBMCs could serve as a diagnostic biomarker for CRC. We also sought to examine the association between *MLH1* methylation and distinct clinical characteristics, including tumor stages and MSI status.

Materials and Method

Blood sample

In this case-control study, blood samples were obtained from a total of 120 participants, including 60 patients diagnosed with sporadic CRC through colonoscopy and confirmed by pathology laboratory results, and 60 ethnicity-matched individuals with negative colonoscopy reports who were referred to Imam Reza Hospital of Mashhad University of Medical Sciences in Mashhad, Iran. All the participants were unrelated, and the healthy controls did not show any symptoms or have any personal or family history of CRC or related tumors. The experimental protocol was approved by Mashhad University of Medical Sciences (The code of ethics number: IR.MUMS.MEDICAL.REC.1402.030).

Before blood sampling, all participants were required to sign informed consent forms after being provided with a thorough explanation of the study. A structured questionnaire was used to collect data on the demographic and clinical characteristics of the participants, including age, gender, height, weight (for calculating body mass index; BMI), and smoking habits. Additionally, details on tumor stage and MSI status were documented based on histological reports. For each participant, 3 ml of venous blood was collected in EDTA-containing tubes and stored at -20°C for further analysis.

PBMCs isolation and DNA extraction

PBMCs were isolated from the peripheral blood specimens using standard density gradient centrifugation with Ficoll-Hypaque solution (Ficoll-Hypaque, Sigma), as previously described.[22] DNA was extracted from PBMCs using a commercial DNA extraction kit (Cinnagen, Iran). The quality of extracted DNA was then verified using the NanoDrop spectrophotometer device and agarose gel electrophoresis.

Methylation-quantification of endonuclease-resistant DNA (MethyQESD)

MethyQESD was used for quantitative methylation analysis of *MLH1*, using the methodology described by Bettstetter et al.[23] This method is a combination of methylation-sensitive and insensitive digestion, followed by quantitative analysis of DNA methylation using real-time PCR. We used two separate sets of samples for our experiment. In the first set, we conducted digestion with the methylation-sensitive endonuclease Hin6I for methylation -specific quantification (MQD), while in the second set, digestion was carried out using methylation-independent endonucleases (XbaI and DraI) for Methylation-Independent Calibrator Digestion (CalD). Consequently, real-time PCR was used to quantify the percentage of methylation using this formula: Methylation % = $E^{\Delta Ct} \times 100$, where ΔCt = Ct Calibrator − Ct methylation quantification (E: PCR efficiency).

Statistics

The statistical analysis was performed by SPSS, Version 25 to evaluate the methylation status of *MLH1* promoter sequences in both the patients and control groups. The receiver operating characteristic (ROC) curves were constructed and the areas under the curve (AUC) were calculated to determine the diagnostic significance of *MLH1* gene methylation in CRC development. The optimal cutoff value for *MLH1* promoter methylation to distinguish between CRC patients and healthy controls was determined using ROC curve analysis. This analysis yielded sensitivity (true positive rate) and specificity (true negative rate) values. Also, we investigated the potential association between *MLH1* methylation level and clinical features using either one-way ANOVA or independent t-test. A *P*-value of less than 0.05 was considered statistically significant.

Results

Demographic and clinical features

The CRC group consisted of 21 females and 39 males with a mean age of 56.80 ± 11.39 years, while the healthy control group comprised 24 females and 36 males with a mean age of 55.26 ± 9.90 years. The age and gender of the CRC patients were similar to those of the healthy participants, indicating proper matching ($P > 0.05$). Besides, there was no significant difference in BMI ($P = 0.859$) and smoking ($P = 0.404$) habits between CRC patients and healthy subjects. In terms of the TNM staging system, 18 (30.0%) of patients were in stage I, 23 (38.3%) were in stage II, 12 (20.0%) were classified as stage III, and 7 (11.7%) were diagnosed as stage IV of the disease. Additionally, 18.3% of all CRC patients tested positive for MSI (Table 1).

MLH1 promoter methylation analysis

Our investigation revealed that the mean levels of *MLH1* promoter methylation in the CRC patients and control participants were 23.40%±16.08% and 10.96%±11.79%, respectively. According to the data in table 2 and figure 1, there is a significant difference in the mean levels of *MLH1* methylation observed in the CRC group compared with healthy individuals ($P < 0.001$). We performed ROC analyses on both the case and control groups, using a fixed cutoff value of 13.12%. This cutoff value yielded a sensitivity of 76.7% and a specificity of 75.0% for distinguishing CRC from normal samples,

indicating appropriate accuracy in diagnosin g CRC from non-CRC individuals (AUC = 0.768, $P < 0.001$, Figure 2).

Moreover, there was a notable difference in *MLH1* methylation levels among different stages of CRC samples, with significantly increased methylation in higher stages ($P < 0.001$). Although, the methylation level was found to be higher in CRC patients with MSI in their tumors (31.58% ± 12.44%), compared with patients without MSI (21.56% ± 16.33%), this disparity did not reach statistical significance ($P = 0.061$). Moreover, there was no significant difference in the mean percentage of *MLH1* methylation between the two groups of CRC patients aged ≤54 and >54, and when stratified by gender ($P > 0.05$). The associations between demographic and clinical characteristics of CRC patients and methylation levels are detailed in table 3.

Discussion

In the present study, we showed that the methylation pattern of *MLH1* promoter sequence in PBMCs of patients with CRC differs significantly from that of normal individuals. Furthermore, ROC analysis demonstrated that using *MLH1* methylation as a biomarker, can be served as a powerful indicator for distinguishing between CRC patients and controls (AUC = 0.768, Figure 2).

Recently, it has been discovered that blood cells such as leukocytes or PBMCs can serve as a novel source of biomarkers for various diseases, including cardiovascular disorders and malignancies.[11] Using this promising approach, scientists tried to identify the epigenetic profile of diseased cells mirrored by these peripheral blood cells. It has been revealed that the methylation levels of genes are dysregulated in the blood cells of cancer patients, mirroring the same changes in tumor cells. This is hypothesized to be mediated by tumor-derived exosomes carrying proteins or regulatory RNAs.[11,24] Previous studies have specified that the methylation profile of multiple genes and loci such as *TUSC3*, *ITGA4*, *MGMT*, *MMP9*, *PD-1*, *PD-L1*, *SEPT9*, *SDC2*, *FOXP3*, *IFNG*, *TFPI2*, *NDRG4*, and *LINE-1*, is altered in PBMCs of patients with malignancies such as breast cancer, hepatocellular carcinoma, CRC, and various other types of tumors.[13,14,17,25-32]

To track the methylation changes in PBMCs of tumor cells, we selected a gene that plays a crucial role in CRC tumorigenesis and progression and is commonly dysregulated in CRC cells, particularly through methylation. *MLH1* is considered as one of the most important DNA MMR genes commonly dysregulated in CRC. *MLH1*, along with *MSH2*, is an independent prognostic and predictive indicator for patients in stages II-III of CRC.[33] *MLH1* dysregulation due to promoter hypermethylation can lead to MSI in CRC tumors.[34] Recent molecular profiling studies on CRC have shown that 75% of *MLH1*-hypermethylated CRC cases displayed MSI status.[35] Previous studies indicate that *MLH1* promoter methylation in sporadic CRC varies from 0.0% to 66.9%.[36-38]

In the present study, we carried out a quantitative analysis of MLH1 promoter methylation in PBMCs of patients with CRC tumors and non-cancerous individuals, for the first time. We demonstrated that the methylation pattern of *MLH1* promoter sequence in PBMCs of patients with CRC differs significantly from that of normal individuals, which is in line with previous studies on CRC tumor tissues indicating hypermethylation of *MLH1*.[37,39] This finding suggests that the methylation profile of PBMCs mirrors that of CRC cells. In the study conducted by Gausachs et al., the methylation analysis of *MLH1* in tumor cells showed a sensitivity rate of 66% and a specificity rate of 96%.[40] In a systematic review and meta-analysis by Li et al., it was determined that the specificity and sensitivity rates of *MLH1* methylation for head and neck squamous cell carcinoma were 95% and 23%, respectively.[41]

In our study, we also observed an association between the methylation level of *MLH1* and the stages of CRC tumors in the patients' group, indicating increased methylation levels in higher tumor stages. However, no association was found between the level of methylation and factors such as age, gender, and MSI status. In contrast with this finding, a meta-analysis by Li et al., revealed a significant association between methylation level of *MLH1* and clinicopathological and molecular characteristics of CRC including gender, tumor location, tumor differentiation, *MLH1* protein expression, and *BRAF* mutation.[39]

Some drawbacks of the present study included the relatively small sample size for effectively detecting *MLH1* methylation levels in PBMCs and the number of evaluated genes. Therefore, further comparative studies with larger sample sizes and multiple genes are needed to corroborate the presented findings.

Conclusion

To the best of our knowledge, this is the first study to investigate the level of *MLH1* promoter methylation in PBMCs, and introduce it as a novel biomarker with high power and accuracy in distinguishing CRC patients from healthy individuals in the early stages of the disease.

Acknowledgments

We would like to thank the Clinical Research Development Center, Ghaem Hospital, Mashhad University of Medical Sciences, for their assistance in this study. This work was supported by Research Project No. 4012305 as an internal residency thesis (By Dr. Farzaneh Yousefisadr).

Authors' Contributions

F.Y: Study design, and reviewing the manuscript; M.M: Study design, Data gathering, drafting and reviewing the manuscript; A.B.N: Study design, data gathering, drafting and reviewing the manuscript; S.S: Study design, data acquisition, data analysis and interpretation, drafting and critical reviewing of the

manuscript; H.M.M: Study design, reviewing the manuscript. All authors read and approved the final manuscript and agreed with all parts of the work in ensuring that any queries about the accuracy or integrity of any component of the work were appropriately investigated and handled.

Funding
None declared.

Conflict of Interest
None declared.

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Table 1. Demographic and clinical characteristics of colorectal cancer patients and healthy controls

Variable	**Case (n = 60)**	**Control (n = 60)**	***P* value**
Sex			
Male	39(65.0%)	36(60.0%)	0.706
Female	21(35.0%)	24(40.0%)	
Age (mean± SD)	56.80 ± 11.39	55.26 ± 9.90	0.433
BMI (mean± SD)	25.11 ± 3.82	25.23 ± 3.34	0.859
Smoker	18(30.0%)	13(21.7%)	
Non-smoker	42(70.0%)	47(78.3%)	0.404
Stage			
I	18(30.0%)	--	
II	23(38.3%)	--	
III	12(20.0%)	--	
IV	7(11.7%)	--	
MSI			
Positive	11(18.3)	--	
Negative	49(81.7)	--	

BMI: Body mass index; MSI: Microsatellite instability; SD: Standard deviation

Table 2. The percentages of methylation in *MLH1* gene in colorectal cancer patients and healthy control groups and their diagnostic value

Group	**AM**	***P***	**Cut-off**	**Sensitivity**	**Specificity**
Case (n = 60)	23.40 ± 16.08	< 0.001*	13.12%	76.7%	75.0%
Control (n = 60)	10.96 ± 11.79				

* *P* value < 0.05; AM: Average methylation

Table 3. The correlation between methylation level of *MLH1* and clinical characteristics in colorectal cancer patients' group

Group	Methylation %	*P* value
Stage (classification 1)		
I	17.95 ± 9.52	< 0.001*
II	16.69 ± 10.93	
III	33.18 ± 18.39	
IV	42.67 ± 18.50	
Stage 2 (classification 2)		
I and II (n = 41)	17.24 ± 10.23	< 0.001*
III and IV (n = 19)	36.68 ± 18.51	
Gender		
Male	18.51 ± 16.99	0.983
Female	23.33 ± 14.63	
Age		
≤54 (n = 26)	26.82 ± 17.81	0.151
>54 (n = 34)	20.78 ± 14.34	
MSI		
Positive (n = 11)	31.58 ± 12.44	0.061
Negative (n = 49)	21.56 ± 16.33	

* *P* value < 0.05; MSI: Microsatellite instability

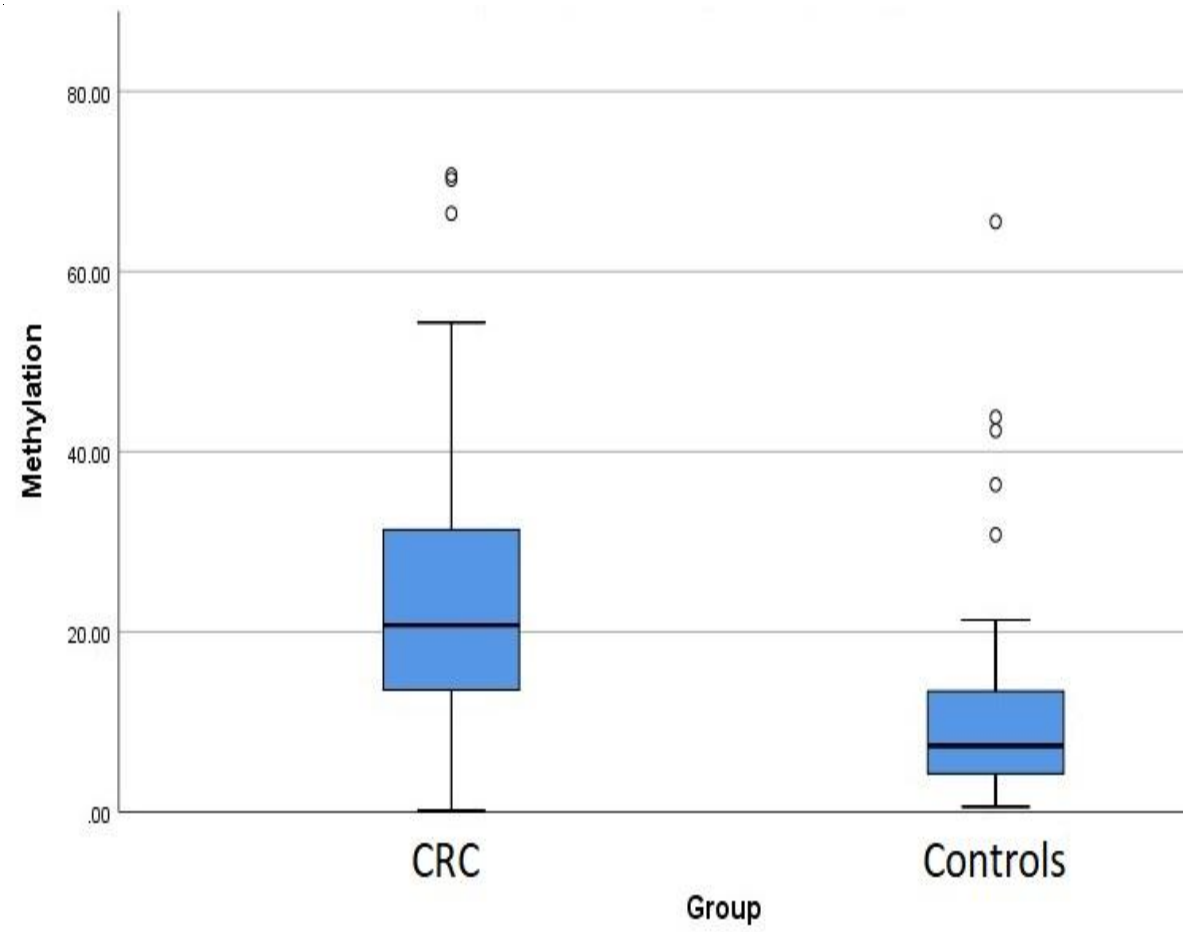

Figure 1. This figure compare the methylation level of *MLH1* promoter between CRC patients and healthy controls.
P <0.001; CRC: Colorectal cancer

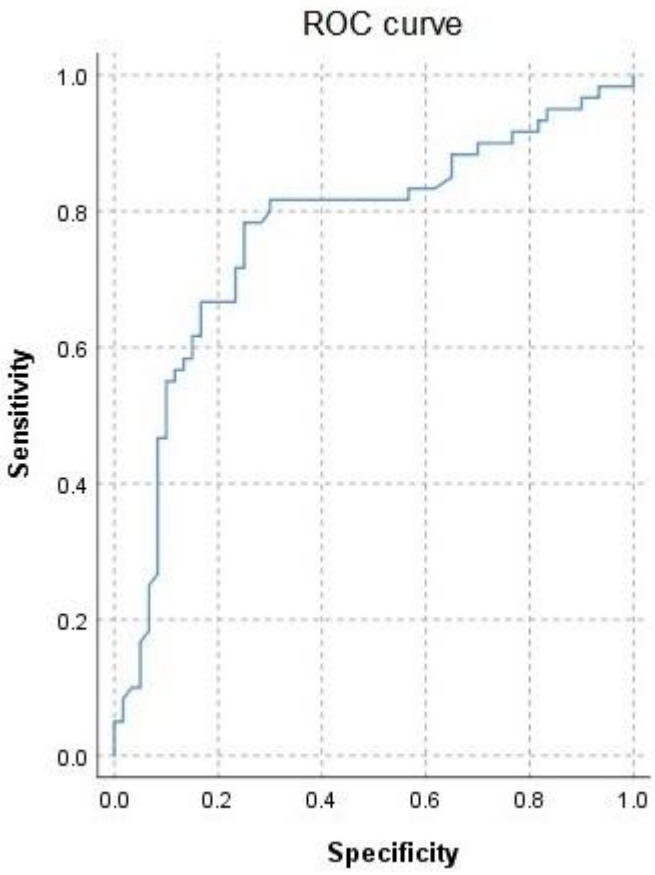

Figure 2. This figure depicts the ROC curves of the methylation level of *MLH1* promoter in patients with colorectal cancer compared with healthy controls.
Area under the curve = 0.768; *P* <0.001; ROC: Receiver operating characteristic