Original
ISSN 2413-0516

The DNA Methylation of the Interleukin-6 as a Biomarker for the Early Detection of Colorectal Cancer

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(Submitted: 27 June 2023 – Revised version received: 13 July 2023 – Accepted: 20 August 2023 – Published Online: 26 December 2023)

Abstract

Objectives: To identify the DNA methylation pattern of the interleukin6 promoter region in colorectal cancer patients and colorectal polys patients that could be a biomarker for early detection of colorectal cancer.

Methods: For this purpose, we examined the DNA methylation pattern of the promoter region of the IL6 specifically 358 bp (–292 to +67) including 7 CpG sites (–228, –213, –163, –119, +10, +19, +25) in a total of ninety samples with thirty samples each for controls, cancer patients, and colorectal polyps using the bisulfite conversion sequencing method.

Result: Our results indicate that CpG +25 and CpG –119 can serve as biomarkers for early detection of colon cancer, showing a significant difference in *P*-values of 0.001 at CpG +25 and 0.004 at CpG –119. The hypomethylation of CpG –119 in cancer groups facilitates the binding of the methyl-sensitive transcription factor Sp1. It enhances the overexpression of IL6 besides hypermethylation of CpG +25 that prevents binding of the methyl-sensitive insulator CTF, unbinding the insulator to the promoter region of the cancer samples makes the promoter region open access to the TFs that enhances overexpression. Furthermore, a remarkable non-recorded SNP at CpG –228 was observed in 98.6% of the enrolled groups.

Conclusion: In the state of DNA methylation, IL 6 could contribute to the onset of colorectal polyps and colorectal polyps cancer due to a significant level of methylation in CpG +25 and CpG –119.

Keywords: Colorectal cancer, DNA methylation, Epigenetic, Hypermethylation, Interleukin 6

Introduction

DNA methylation is an epigenetic modification regulates the gene expression that affects phenotype without changing the genotype. This process involves the enzyme DNA methyltransferase, which adds a (methyl group CH3) to the cytosine in CpG sites to form 5-methylcytosine by the methyl donor S-adenosyl-l-methionine. This enzymatic addition of methyl groups is often referring as the writer of DNA methylation.[1] DNA methylation is primarily maintained by the enzyme (DNA methyltransferase 1 DNMT1) which ensures the replication of DNA methylation patterns during cell division. besides, de novo DNA methylation establishes a new methylation pattern by the enzymes (DNMT3A and DNMT3B). These enzymes are responsible for adding methyl groups to previously unmethylated cytosine residues.[2]

DNA methylation of the promoter region which is a motif for the transcription factors enhances overexpression of oncogenes such as growth factor genes involved in cell cycle regulation or decreases expression of onco-suppressor genes responsible for apoptosis and DNA repair.[3] DNA methylation suppresses the binding of the methyl-sensitive transcription factor while the methyl-resistant transcription factors accessing DNA methylation motif.[4]

The promoter region of the Il6 contains a motif for the Methyl-sensitive transcription factor SP1 bind to the DNA sequence CCCGCCC,[5] and methyl-sensitive CREB binds to the DNA sequence GACGTCA that enhance the expression.[4] In contrast, the CTF zinc finger protein binds to CCCTC is a methyl-sensitive that serves as an insulator and suppression transcription.[6]

Colon cancer is the second deadly cancer in females, following breast cancer, and the third prevalent cancer in males, ranking after lung and prostate cancer which primarily affects older adults that may correlate with DNA methylation of some oncogenes such as inflammatory cytokines that enhance cell surviving, one of the most important cytokines is Interleukin 6 (IL6) which is a pro-inflammatory cytokine located on chromosome 7p15.3. Overexpression of IL6 has been observed in various tumors, influencing cancer cells through multiple signaling pathways by suppressing apoptosis and enhancing cell survival, proliferation, angiogenesis, invasiveness, and metastasis. As such, IL6 serves as a valuable tumor marker for monitoring the metastasis of cancer.[7]

The objective of this study was to evaluate the methylation patterns of the IL6 gene promoter region in individuals with colorectal cancer and colorectal polyps. Establishing an effective biomarker for the early detection of colon cancer.

Materials and Methods

In this study, ninety cases were taken into consideration and categorized in to the followings: thirty samples from colon cancer patients from Hewa Hospital in Sulaimani, thirty colorectal polyp's samples from the GIT Center of Sulaimani Hospital and the reminding 30 samples considered as a control group from private laboratory. The sample collection period spanned from August 27th, 2021, to February 13th, 2022.

All participants in the control group underwent tests for inflammatory biomarkers, including C-reactive protein (CRP), fecal calprotectin, and erythrocyte sedimentation rate ESR, which all yielded negative results. The colorectal polyps and colon cancer patients were confirmed through histopathological examination done by the histopathological laboratory of the GIT center, the size of the tumor were determined by CT scan and blood from cancer group were collected from the patients before undergoing chemotherapy.

Besides age and gender, we collected several important patient histories for our study cohorts. These include cancer stage, cancer size, location of cancer, blood sugar levels, liver function tests, complete blood count (CBC), cancer biomarkers, and smoking status.

DNA Extraction

The ReliaPrep™ Blood gDNA Miniprep System kit (Promega, USA) was used for extracting DNA from the blood. The bisulfite conversion sequencing method was employed. The entire genome underwent treatment with the MethylEdge® Bisulfite Conversion System kit (Promega/USA) to convert unmethylated Cytosine to uracil, following the manufacturer's instructions.

Detecting the Promotor Region

To detect the promoter region, we used software such as RefSeq, GenBank from NCBI, Ensemble, and the Eukaryotic Promoter Database (EPD). It was crucial to identify the specific sequence of cis-acting elements such as the TATA box, GC box, and CAAT box to highlight the promoter region and locate CpG within it.

Primer Designing

Methyl primers were designed using the Zymosearch software (https://zymoresearch.eu/pages/bisulfite-primer-seeker). The 25bp forward primer 5′TAAAGTGTTGAGTTATTAATAAAAG3′ and the 24 bp reverse primer 5′TCATAACTAAACTCCTAAAAAAAA3′ used for amplifying 358 bp from −292 to +66 containing 7 CpG sites (−228, −213, −163, −119, +10, +19, +25). The primer dimer avoided by checking primers using Eurofins software (https://eurofinsgenomics.eu/en/ecom/tools/oligo-analysis).

Identifying Transcription Factors

To identify transcription factors within or near CpG of the IL6 promoter region we utilized five different software tools: Alibaba software, Softberry software, Tfsitescan software, Pwmtools software, and PROMO Software Version 3.0.2.[8] The methyl-sensitive transcription factor SP1 binds to the DNA sequence CCCGCCC, the methyl-sensitive CREB binds to the DNA sequence GACGTCA, Additionally, the methyl-sensitive CTCF binds within the DNA sequence CCCTC.

PCR Amplification

The 358 bp IL6 promoter region of all samples were amplified by PCR. As a control, the converted methylated human control (Promega/USA) was utilized. The GoTaq® Hot Start Green Master Mix (Promega/USA) along with a 25 bp forward primer (5′TAAAGTGTTGAGTTATTAATAAAAG3′) and a 24 bp reverse primer (5′TCATAACTAAACTCCTAAAAAAAA3′) using the hot start technique. The PCR cycles consisted of an initial enzyme inactivation at 94°C for 5 min. (1 cycle), followed by 35 cycles. Each cycle involved denaturation at 94°C for 1 min., annealing at 50°C for 1 min., and extension at 68°C for 1 min. Finally, there was a final extension step at 68°C for 7 minutes.

To assess the quality of the PCR product, the safe dye Diamond nucleic acid dye from Promega (USA) was employed. Agarose gel 1.5 g was prepared, and 100bp of the DNA ladder from Biotech (India) was loaded onto the gel. The gel was then visualized and documented using the Accuris SmartDoc system from Accuris (USA).

Sequencing the DNA Product

Following PCR purification, 20 µl of working stock primers and 25 µl of the amplicon were sent to Magrogen (Korea) for downstream applications using Sanger sequencing. The sequenced data were viewed using FinchTV, SnapGene, and BioEdit software viewer to examine the chromatogram results. Additionally, the online BLAST software, specifically the Needleman-Wunsch Global Align Nucleotide Sequences algorithm utilized to align the sequenced gene.

The Bioinformatics DNA methylation analyzer QUMA software (http://quma.cdb.riken.jp/) was employed as a DNA methylation analyzer to estimate the density of methylated and unmethylated CpG sites, calculated as the number of converted nucleotides per fragment.

Results

Patient History

Our study included 30 cancer patients with colon cancer aged between 32 to 92 years old. Among them, there were 12 females and 18 males and cancer sites were the sigmoid, rectum, ascending colon, transverse colon, and descending colon. Additionally, we included 30 samples from patients with colorectal polyps aged between 17 to 76 years old, with 17 females and 13 males. Histopathological results confirmed the absence of malignancy in these cases. Furthermore, we included 30 control samples with no history of polyps or cancer. These control samples were aged between 19 to 82 years old, with 18 males and 12 females.

In our study, we made several significant findings. Firstly, we observed platelet distribution width (PDW) levels were elevated in 80% of the cancer samples, with a mean value of 17.6 ± 2.4. Furthermore, we found colon cancer patients exhibited elevated levels of random blood sugar, with a mean value of 135 ± 6.3 indicating positive relation between colon cancer and abnormal blood sugar levels. Moreover, gender was found to be correlated with the overexpression of IL6 expression. Specifically, 85.3% of the methylated CpG −119 sites in the control group were found to be male. This suggests a gender-based influence on the expression of IL6. Additionally, hypermethylation of CpG +25 was found to be correlated with the erythrocyte sedimentation rate (ESR) level with mean 37 ± 4.6.

Sequence Alignment

The Needleman-Wunsch Global Align Nucleotide Sequences software was employed to highlight the conversion of all unmethylated CpG sites to uracil during the bisulfite treatment and subsequent sequencing. Samples with less than 96% similarity in alignment were excluded from further analysis. This step ensured that the analyzed samples maintained a high degree of similarity in the alignment. The methylation level of all seven CpG sites and the number of unmethylated and methylated CpG sites were analyzed as shown in Table 1.

The QUMA software was used to detect the methylation level. Hypermethylation observed at CpG +25 was 38% in the cancer group whereas in the control group was 0%, and in polys groups was 13.2%. In contrast, hypermethylation observed at CpG −119 in the control group was 23.3%, whereas in both

cancer and polyp's groups was 0%. On the other hand, the hypermethylation in CpG +19 was observed just in the polyps group was 4%, also the level of methylation at the CpG –228 was 5% just in the polyps group. The remaining CpGs (–213, –163, and +10) showed hypomethylation (0%) in all three groups just in the control group methylation levels were 3% at CpG +10 as shown in Figure 1.

Statistical Analysis Using T-test for Detecting Significantly the DNA Methylation

For detecting the comparison between groups, the T-test used a paired-samples t-test (statistical package for social science) version 24.0.

A significant difference was observed in hypermethylation between the control group and the cancer group in CpG-119 the P-value was 0.0009 as shown in Table 1 also the *P*-values 0.0009 at CpG-119 was significant compared to the control group with the colorectal polyps shown in Table 2.

In addition, a significant difference was observed in the methylation patterns between the cancer group and the control group at CpG +25. The cancer group showed hypermethylation the *P*-value was 0.004 as shown in Table 3.

Table 1. **Summary and statistics of overall sequences in the control, cancer and polyp's groups**

DNA methylation summary over all sequences			
Unmethylated CpGs	Cancer	93.4%	193 sites
	Control	91.4%	192 sits
	Polyps	78.57%	165 sites
Methylated CpGs	Cancer	2.38%	5 sites
	Control	3.81%	8 sites
	Polyps	1.9%	4 sites
Statistics about CpGs analyzed over all sequences			
CpGs analyzed	Cancer	94.29%	198 sites
	Control	95.71%	201 sites
	Polyps	80.48	196 sites
CpGs not analyzed	Cancer	5.71%	21 sites
	Control	4.2%	9 sites
	Polyps	19.52%	41 sites

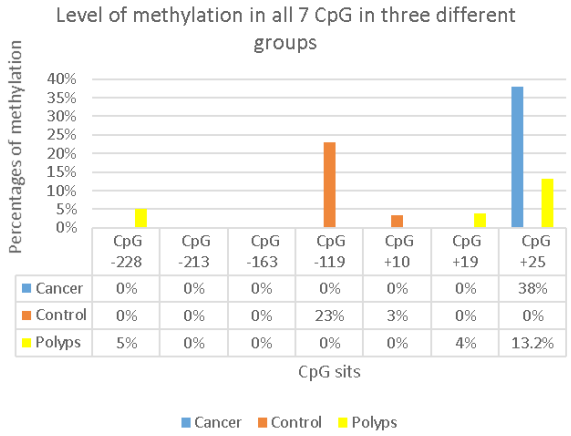

Fig. 1 **Comparison between the level of methylation in each 7 CpG site between all groups.**

Table 2. **The correlation between the methylation level of CpG-119 of the control group compare with colorectal polyps**

	control CpG -119	polyps CpG -119
Mean	0.266666667	0
Variance	0.202298851	0
Observations	30	30
Pooled variance	0.101149425	
Hypothesized mean differ	0	
df	58	
t Stat	3.247376564	
P(T< = t) one-tail	0.000969114	
t Critical one-tail	1.671552762	
P(T< = t) two-tail	0.001938228	
t Critical two-tail	2.001717484	

Table 3. **The correlation between the methylation level of CpG +25 of the control group compare with cancer group**

	Cancer group CpG +25	Control group CpG +25
Mean	0.233333333	0
Variance	0.185057471	0
Observations	30	30
Pooled Variance	0.092528736	
Hypothesized mean differ	0	
df	58	
t Stat	2.970873097	
P(T< = t) one-tail	0.002157755	
t Critical one-tail	1.671552762	
P(T< = t) two-tail	0.00431551	
t Critical two-tail	2.001717484	

Polymorphism within the CpG Site

According to our study, we investigate a novel polymorphism C/G at CpG –228 in 98.6% of our samples at DNA site 22.72.973, which is not recorded in NCBI.

In addition, several SNPs were detected at the CpG sites. A polymorphism C/- with rs1784021731 was detected at CpG +25 where a cytosine deletion occurred at DNA site 22.727.225. Another SNP, rs1455154786, was detected at CpG -19, resulting in a cytosine deletion at DNA site 22.727.218. Additionally, rs941000565 with the SNP C/A was detected at CpG –163 at DNA site 22.727.038. Furthermore, the SNP C/T with rs178401730 was found at DNA site 22.726.986 in nearly all samples. In the polyps group, in our population, the SNPs rs1460286581 and rs1253413871, with C/T and C/G variations, respectively, were detected specifically at CpG +10 and CpG –119 as shown in Figure 2.

Polymorphism in non-CpG sites

Polymorphisms were detected at –174 bp (rs1800795) with a C/G SNP at the DNA site 22.727.027 on chromosome 7. The

levels of this polymorphism were found to vary among groups. In the cancer group, it was detected in 70% (21 out of 30) of the samples, in the control group it was found in 66% (20 out of 30) of the samples, and in the polyps group, it was observed in 73.3% (22 out of 30) of the samples as shown in Figure 3.

Discussion

DNA methylation is an epigenetic mechanism that regulates gene expression and is involved in various cellular processes. It is facilitated by DNA methyltransferase enzymes, which add a methyl group to the cytosine in CpG sites. Abnormal methylation correlated with oncogenes.[9]

Colorectal cancer is a cancer-related mortality and inflammation has been found to have a positive correlation with the onset of cancer, study by[10] provided evidence that inflammation can trigger the growth of cancer cells, suggesting a potential link between autoimmune diseases and the development of cancer. Specifically, patients with inflammatory bowel disease have been found to be more susceptible to colon cancer. The study by[11] reveals that approximately 60–65% of colon cancers are classified as sporadic, indicating that they occur without a clear hereditary or familial link. Importantly, the study emphasizes that inflammation plays a significant role in increasing the incidence of colon cancer. The study by[12] suggests that ulcerative colitis is likely involved in the progression of colorectal cancer. It highlights the importance of understanding the specific genes and regions affected by hypomethylation, which is an ongoing area of research. Such knowledge can provide valuable insights into disease mechanisms and potentially identify therapeutic targets for intervention.

To investigate the correlation between inflammation and the progression of colon cancer, our study focused on the level of DNA methylation of IL6 cytokine which is involved in various autoimmune diseases. Based on preliminary evidence, there are indications that IL6 is implicated in the development of cancer. A study by[13] demonstrated a positive correlation between elevated levels of IL6 and the stages of colorectal cancer.

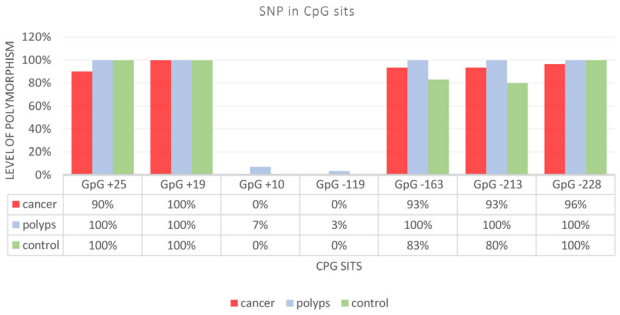

Fig. 2 **Comparison between level of SNPs in all groups in CpG among promoter region of IL6.**

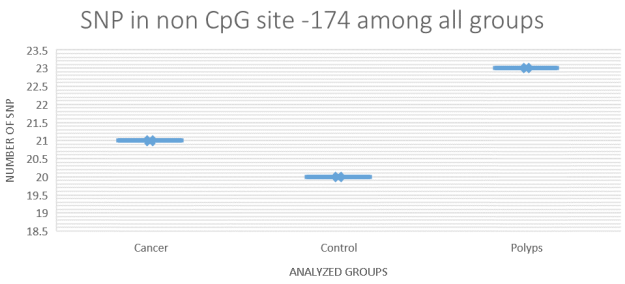

Fig. 3 **Different level of the polymorphism at –174bp in all groups.**

In our study, we utilized the bisulfite sequencing technique to evaluate the methylation status of seven CpG sites (+25, +19, +10, –119, –163, –213, and –228) within a 358bp region in the promoter region of IL6. A total of 90 samples were analyzed to investigate the methylation levels at these sites as shown in Table 1.

Our results indicate that both CpG –119 and CpG +25 can serve as potential biomarkers for the early detection of colon cancer. Remarkably, we observed a significantly hypermethylation in CpG –119 within the control group compared to both the cancer group and the polyps group, with a *P*-value of 0.001 as shown in Table 1 and Table 2. Obviously, CpG –119 is within motif for the methyl-sensitive transcription factor Sp1, which has a preference for unmethylated CpG sites and overexpression of IL6 due to the binding to CpG –119 indicating that over-expression of IL6 occurs in cancer groups due to hypomethylation of CpG –119 Our result shows that hypomethylation of CpG –119 has the potential to be a biomarker for early detection of colon cancer. Our study along with the previous study by[14] demonstrated the impact of IL6 hypomethylation on oral cancer however this study did not detect methylation levels at specific sites and there was not any previous study parallel to our study to make the comparison that may make our study a novel study according to detecting methylation level of the IL6 at specific CpG.

Additionally, we observed a significant difference in hypermethylation of CpG +25 between the cancer group and the control group *P*-value was 0.004 as shown in Table 3 which is within the motif of the methyl-sensitive CTF which is the insulator site CTCF. Hypermethylation in the cancer group at CpG +25 indicates that the methylation prevents binding of the insulator and the promotor region in open access to the TFs, indication difference between cancer group with control group and Polyp's group which was related with high level of the ESR indication that inflammation may trigger the onset of the cancer.

Detecting hypomethylation with methylation level 0% at CpG –163 which is within the motif of the transcription factors CRE in all groups indicates that the promoter region of the IL6 was open access for binding the methyl-sensitive CRE in all groups, however, transcription depends on hypermethylation of the CIF motif that suppresses expression.

Furthermore, the CpG +19 methylation level in the polyps group was 4%, whereas both the cancer group and the control group displayed a hypomethylation level of 0%. Our findings were parallel with a previous study by[15] that demonstrated hypomethylation in the promoter region of both rheumatoid arthritis (RA) patients and the control group.

Hypomethylation was observed in CpGs +10 across all groups, with a methylation level of 0% groups, just in the control group methylation levels were 3%. This finding is consistent with a study conducted by[16] which also reported hypomethylation in the promoter region of depressed patients compared to the control group. However, these studies only assessed the level of methylation using the quantitative pyrosequencing technique.

The methylation level in CpG –228 was 5% just in the polyps group while hypomethylation observed it was 0% in the cancer group and control group also hypomethylation in CpG –213 was observed in all given groups.

In addition to DNA methylation, other factors may play a role in colon cancer. One such factor is the platelet distribution width (PDW), which was found to be elevated in 80% of cancer samples, with a mean of 17.6 ± 2.4. PDW represents the variation in platelet size and is associated with platelet activation seen in many types of cancer. Research conducted by[17] demonstrated that an increased PDW level could serve as a biomarker for the early detection of laryngeal cancer. Furthermore, a study by[18] indicated a positive correlation between PDW and the presence of sepsis in colorectal cancer patients. Conversely, a study by[19] suggested that a high PDW level is associated with the prognosis and progression of gastric cancer. However, Colon cancer is primarily diagnosed through specific screening tests like colonoscopy, fecal occult blood tests, or tumor markers. These tests are more reliable and accurate in detecting colon cancer compared to using PDW as a diagnostic marker. Cancer-related changes in the bone marrow, systemic inflammation, and interactions between cancer cells and platelets can affect platelet size and its distribution. Therefore, in some cases, increased PDW levels have been reported in patients with advanced stages of colon cancer or as part of a systemic inflammatory response.

There is evidence suggesting a potential correlation between blood sugar levels and colon cancer, although the relationship is complex and not fully elucidated. In our study, we observed elevated random blood sugar levels in patients with colon cancer, with a mean of 135 + 6.3. This finding indicates that blood sugar may contribute to the development of colorectal cancer, which aligns with a study conducted by[20] demonstrating that high blood sugar levels serve as a risk factor for colon cancer.

In addition to DNA methylation analysis, our sequence analysis revealed the presence of several single nucleotide polymorphisms (SNPs) in the promoter region, including both CpG and non-CpG sites. Notably, we discovered a novel polymorphism, in 98.6% of given groups specifically a C/G variation at CpG –228 at DNA site 22.72.973 which has not been previously documented in the NCBI database.

Furthermore, our analysis identified several SNPs at the CpG sites, including polymorphisms such as rs1784021731, rs1455154786, rs941000565, rs178401730, rs1460286581, and rs1253413871, as shown in Figure 2. We did not observe significant differences between the groups concerning these SNPs. However, it is noteworthy that a small number of individuals in the polyps group exhibited the presence of SNPs rs1460286581 and rs1253413871 exclusively. These polymorphisms have the potential to alter the CpG sites and subsequently impact the methylation levels and the binding of transcription factors.

We observed a significant SNP, namely –174 G/C with rs1800795, located at DNA site 22.727.027. Interestingly, this SNP was detected in non-CpG sites in 70% of the groups examined, with slight variations between the groups as depicted in Figure 3. Result of our study was parallel to a study conducted by[21] suggested a relationship between the –174 G/C SNP and type 2 diabetes. However, in our study, we found no indication that this SNP (–174 G/C) is associated with colon cancer.

Conclusion

The DNA methylation patterns of IL6 were significantly different between the healthy group and the colorectal cancer group at CpG –119 and CpG +25, suggesting that they can serve as biomarkers for the early detection of colon cancer. Based on our results, we can conclude that inflammatory cytokine IL6 is overexpressed in the cancer group and polyps group, likely due to hypomethylation at CpG –119 in the cancer group, which is a motif for the transcription factor Sp1 that enhances transcription and hypermethylation of CpG +25 which prevent binding of the insulator CTF.

Acknowledgment

I would like to express my special thanks to all who helped me throughout this work.

Funding

This study did not receive any funding in any form.

Conflict of Interest

Fortunately, there was no conflict with any research centers or institutes.

Author Contributions

Both authors shared roles in the laboratory collecting samples, manuscript writing and statistical analysis.

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