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Aberrant methylation detection of the hMLH1 promoter and the IGF2 DMR region using methylation-sensitive high resolution melting (MS-HRM) in colorectal cancer

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ABSTRACT

Epigenetic alterations and gene mutations causing inactivation of tumor suppressor genes and activation of oncogenes can regulate signaling pathways contributing to colon cancer formation. Many genes have been reported to be aberrantly methylated in the CRC genome, and it is likely that only subsets of these genes are important in the pathogenesis of colorectal tumors. We applied the methylation-specific high resolution melting (MS-HRM) technique to study methylation of the IGF2 DMR and the hMLH1 gene promoter in 60 colorectal cancer and adjacent normal tissues of same patients compare to 20 normal tissue samples. In this study, 5 of the 60 colorectal cancer samples (8.3%) were found to be methylated at the hMLH1 promoter region and 32 of the 60 colorectal cancer samples (53.3%) were found to be hypomethylated at the IGF2 DMR region. Adjacent normal tissues were unmethylated for hMLH1 and 5% showed hypomethylation for IGF2 DMR. There was significant correlation between aberrant methylation of hMLH1 and IGF2 DMR with tumor location (p=0.002, p=0.026 respectively). In addition, a tendency of association between IGF2 DMR hypomethylation and age (p=0.06) was observed. We demonstrated effectiveness of the MS-HRM technique to analyze methylation of IGF2 DMR and hMLH1 promoter region and methylation significantly correlated with tumor location in colorectal cancer patients.

Keywords: IGF2, hMLH1, Colorectal Cancer, Promoter methylation, Methylation-sensitive high resolution melting (MS-HRM).

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Introduction

Colorectal cancer (CRC) is a leading cause of cancer deaths worldwide and also a significant increase in CRC incidence has been reported over the last decade in Iran (1-3). It is now accepted that epigenetic changes and gene mutations cooperatively contribute to normal cell transformation (2). Epigenetic alterations and gene mutations causing inactivation of tumor

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suppressor genes and activation of oncogenes can regulate signaling pathways contributing to colon cancer formation (4). In the colon cancer genome, many genes are aberrantly methylated instead of mutated, and it seems that aberrant methylation is the predominant mechanism observed for the silencing of many genes in cancer. It is likely that only a subset of genes aberrantly methylated in the CRC genome are important in the pathogenesis of colorectal tumors (2). Epigenetic alterations and DNA methylation signatures can be used as clinical biomarkers for detection, diagnosis, prognosis and monitoring in CRC (2, 5).

Insulin-like growth factors (IGFs), including IGF1 and IGF2, are important in regulating essential cellular functions (6). IGF dysregulation is correlated with a number of human diseases including cancer (7). The IGF2 gene is expressed mainly from the paternal allele and is located on chromosome 11p15 within a cluster of imprinted genes (8). Imprinted genes are associated with CpG rich regions that have allele-specific DNA methylation known as differentially methylated regions (DMRs). It has been reported that IGF2 DMR hypomethylation is highly prevalent in cancer and it is found in 80% of colorectal tumor tissues (9). It was indicated that, IGF2 DMR hypomethylation has diagnostic potential for colon cancer and suggested as a useful indicator of CRC(9).

In addition, function of some genes such as hMLH1 is critical to the maintenance of DNA stability in that epigenetic alterations can affect their functions (10). MLH1 gene is a part of the DNA mismatch repair (MMR) system and it has been reported that its promoter methylation can contribute to tumor initiation and progression (10, 11). Microsatellite instability is a feature of tumors with an MMR defect and these tumors are termed MSI. This feature is found in about 15% of colorectal, gastric and endometrial tumors (12). Dysfunction of the DNA mismatch repair system can occur mainly through the CpG methylation-mediated silencing of the hMLH1 gene (13, 14). It has been shown that promoter methylation of this mismatch repair gene can occur at a frequency of 53.4% in malignant colorectal tumors (15).

The method used in this study is based on methylation-specific high resolution melting (MS-HRM), a technology initially developed for genotyping studies (16). The principle behind MS-HRM is bisulfite-treated DNA templates with different methyl cytosine contents. Methylated DNA and unmethylated DNA can acquire different sequences after bisulfite treatment resulting in PCR products with markedly different melting profiles (17). In this technique, the melting profiles of PCR products from unknown samples compare with profiles specific for PCR products derived from methylated and unmethylated control DNAs. We were able to detect extent of methylation with high sensitivity in the screened samples by comparing the melting profiles of unknown samples with the profiles of fully methylated and unmethylated references (17, 18).

In this study we applied the methylation-sensitive high resolution melting (MS-HRM) technique to assess methylation of IGF2 DMR and hMLH1 gene promoter in sporadic CRC tissues of Iranian patients.

Materials and Methods

DNA sample collection and controls

Sixty colorectal carcinoma and adjacent normal tissue specimens, in addition to 20 normal tissue samples, were collected from Imam Khomeini and Firoozgar hospital, Tehran, Iran. Written informed consent was obtained from all patients. Tumor samples were frozen immediately and then stored

at -80°C. All tissue specimens were confirmed histologically. The demographic and clinical characteristics of subjects are shown in Table 1.

Table1. Clinical characteristics of subjects

	Adenocarcinoma	normal
Median age (range), y	57.7(28-88)	44.6(24-60)
Sex (male/female)	(25/35)	(9/11)
Location of tumor (proximal/distal/unknown)	(6/45/9)	
T Stage T1–T2/T3/T4	(17/34/7)	
Grade Differentiation I/ II /III	(33/22/2)	

DNA was extracted from samples by using the DNeasy Tissue Extraction Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. EpiTect methlyated and unmethylated control DNA (100 ul) (Qiagen, Germany) was used as reference for melting experiments. To create a range of methylated and unmethylated allele dilutions, the above two controls were mixed in 25, 50, 75 and 100% methylated to unmethylated template ratios. These standards were included in each experiment.

Bisulfite modification of genomic DNA

We used Epitect Bisulfite Kit (Qiagen, Hilden, Germany) for bisulphite modification of DNA. One µg of genomic DNA extracted from tumor and normal adjacent colorectal tissue specimens were subjected to bisulfite treatment and all the modification reactions were performed according to the manufacturer's instruction. The eluted DNA (40µl volume) was used for HRM analysis.

HRM Analysis

PCR amplification and high resolution melting analysis was carried out in a Rotor-Gene 6000 (Corbett Research, Sydney, Australia). The sequence of the primer used is shown in Table 2.

Table 2. Summary of primer sequences for MS-HRM

Primers	Sequence (5'-3')	(bp)
hMLH1-F	AGTTTTTAAAAACGAATTAATAGGAAGAG	82
hMLH1-R	ACTACCCGCTACCTAAAAAAATATAC	
IGF2-F	TTTAGGAGGGTTAGGTTATAGTTG	105
IGF2-R	TCTATACTATAAAACTTCCAAACAAAC	

The Melt Doctor Master Mix (ABI) containing SYTO-9, an intercalating dye, was used. The reaction mixture consisted of 2 µl (theoretical concentration 12.5ng/ µl) of bisulphite-modified template, and 10 µl Melt Doctor Master Mix, 10 pmol/ µl of each primer in a final volume of 20 ml. The reaction cycle started with one cycle of 95°C for 5 min, followed by 45 cycles at 95°C for 10 sec, 30 s at the primer annealing temperature and 10 s at 72°C. MS-HRM analyses were performed at temperature ramping and fluorescence acquisition settings recommended by the manufacturer (ramping from 60 to 85°C, rising by 0.1°C/2s). All reactions were performed in duplicates. A standard dilution series was run to assess the quantitative properties and sensitivity of the each assay. We assessed the Cycle threshold (Ct) values, end point fluorescence level, amplification efficiency and specificity of each sample because the quality of the MS-HRM results was highly dependent on the quality of the real-time amplification products. Using software provided with the Rotor-Gene 6000, the melting curves were normalized by calculation of the 'line of best fit' in between two normalization regions before and after the major fluorescence decrease representing melting of the PCR product. Direct comparison of samples that have different starting fluorescence levels was allowed with this algorithm.

Validating methylated and unmethylated samples by DNA sequencing

The PCR product of samples showing methylated and unmethylated pattern in HRM analysis were cloned into the P T Z 5 7 R / T vector using a TA Cloning kit (Fermentas) after purification. Five

clones were sequenced per sample using M13 universal primers by ABI 3130 automated sequencer Applied Biosystems (Macrogen Korea).

Statistical Analysis

Using the SPSS software (SPSS Inc, Chicago, I11, version 16.0), statistical analysis was performed. Associations between methylation of loci and clinicopathological parameters were analyzed using the Chi-square test. A p-value less than 0.05 is indicated as statistically significant.

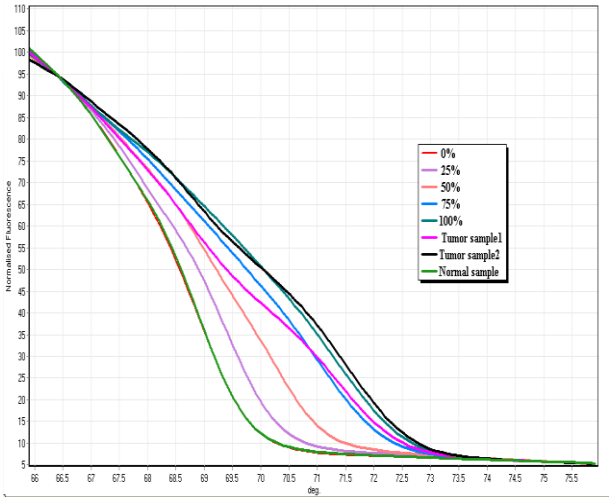
Results

The sensitivity of the hMLH1 and IGF2 MS-HRM assay

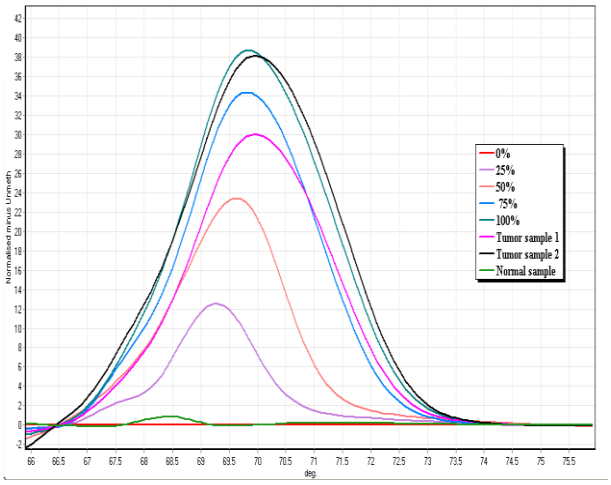
The sensitivity of the hMLH1 and IGF2 MS-HRM assay was tested using the consistency of normalized melting profiles derived from different ratios of methylated and unmethylated control DNA: 0, 25, 50, 75 and 100% methylated. The hMLH1 and IGF2 MS-HRM assay amplified a fragment of 82 bp and 105 bp, respectively. The inclusion of a limited number of CpG dinucleotides into the primer sequence enabled conditionally selective binding of the primer to the methylated sequence.

Methylation status of hMLH1 and IGF2 in the samples

We applied MS-HRM for analysis of MLH1 promoter and IGF2 DMR methylation in 60 colorectal tumor specimens and normal adjacent tissue. Normally, the MLH1 promoter is not expected to be methylated and any methylation of this promoter is abnormal, but low methylation level is not considered biologically significant. Thus, only samples with >25% relative to control were statistically significant. In 5 (8.3%) out of 60 tumor tissue, MLH1 promoter methylation >25% was identified (Figure 1).



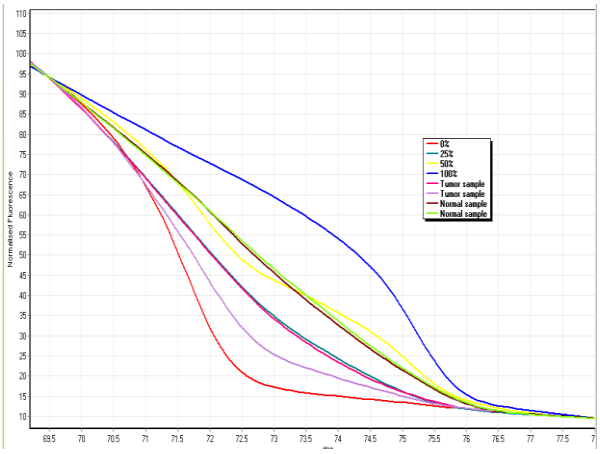
A



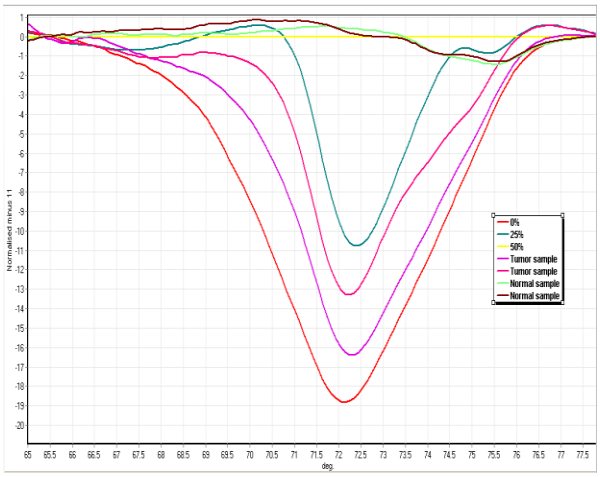
B

Figure 1. MS-HRM analysis of the methylation status of the MLH1 gene in the colorectal cancer and normal samples. (A) Results of the MLH1 MS-HRM assay for tumor and normal samples compared to the dilution standards. Tumor samples in contrast with normal samples showed methylation. (B) Differential graph of fluorescence signal of each sample was normalized against the unmethylated control.

Normally, IGF2 DMR methylation level is 50% and less than 50% methylation in this region, relative to controls, is abnormal. In 32 (53.3%) out of 60 tumor tissue, IGF2 DMR methylation less than 50% was identified (Figure 2).



A



B

Figure 2. MS-HRM analysis of the methylation status of the IGF2 gene in the colorectal Cancer and normal samples. (A) Results of the IGF2 MS-HRM assay for Tumor and normal samples were compared to the dilution standards. Tumor samples showed hypomethylation in contrast with normal samples that were 50% methylated. (B) Differential graph of fluorescence signal of each sample was normalized against the 50% Methylated control.

We identified that samples with methylation values >25% as methylated samples for MLH1 promoter and samples with methylation values less than <50% as hypomethylated samples for IGF2. None of the normal adjacent tissue showed methylation for MLH1 but 5% of normal adjacent tissue showed hypomethylation for IGF2. In contrast, in 20 normal tissue samples, none was showed aberrant methylation for MLH1 and IGF2.

Bisulfite DNA sequencing

To confirm information about methylation pattern of the MLH1 gene promoter and the IGF2 DMR, an 82 bp fragment of the MLH1 promoter region and a 105 bp fragment of the IGF2 DMR in normal tissue and colorectal carcinoma samples, containing 5 CpG and 6 CpG dinucleotides respectively, were sequenced. Sequencing provided further proof that all MS-HRM detected methylated samples for the MLH1 gene promoter demonstrated fully methylated pattern (Figure 3). Less than half of the clones related to hypomethylated IGF2 DMR demonstrated methylated pattern upon sequencing. In normal samples, 50% of clones were methylated and 50% were unmethylated.

Association between methylation and clinicopathological variables

The association between methylation status of hMLH1 and IGF2 DMR and clinicopathological variables are summarized in Tables 3 and 4. There is significant association between CRC and aberrant methylation of hMLH1 (p=0.032) and IGF2 DMR (p=0.0001). No significant correlation was found between hMLH1 methylation and any of the clinical features except a statistically significant correlation with tumor location (p=0.002). Also, no significant correlation was observed between IGF2 DMR hypomethlation and patient gender, tumor differentiation and Duke's stage (p=0.94, 0.38, 0.41). However, there was significant correlation with tumor location (p=0.026) and a tendency of association with age (p=0.06) was observed.

AGTTTTTAAAAACGAATTAATAGGAAGAG**CG**GATAG**CG**ATTTTTAA**CGCG**TAAG**CG**
TATATTTTTTTAGGTAGCGGGTAGT

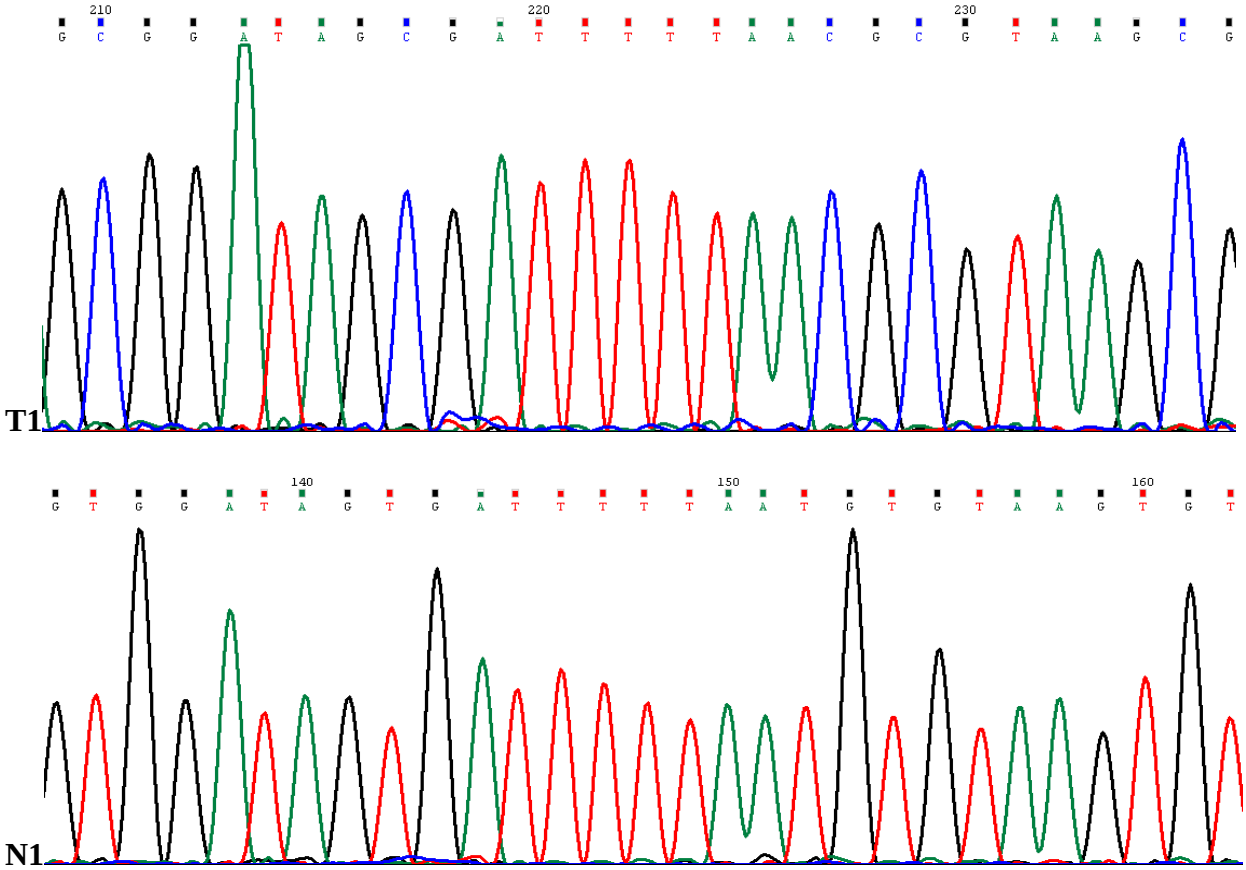


Figure 3. Bisulfite sequencing. A 82bp region of hMLH1 promoter with 5CpG dinucleotides were assayed by bisulfite sequencing. An example of a fully methylated hMLH1 CpG island in sample T1 (top) and an unmethylated CpG island in sample N1 (bottom) can be seen as determined by bisulfite sequencing.

Discussion

It seems that aberrant methylation of genes is a common molecular mechanism for silencing tumor suppressor genes and can contribute to cancer formation through the transcriptional suppression of these genes. In fact, it seems that in genome of colon cancer, for the silencing of many genes, aberrant methylation may be the only mechanism and many more genes are affected by aberrant methylation than by mutations in cancer (19, 20). Previous studies primarily used methylation specific polymerase chain reaction (MSP) based assays. The result of MSP is qualitative. Due to the qualitative nature of the assay, discrimination between low levels of methylation from high levels of methylation by MSP is not possible. Previously, quantitative DNA methylation analysis has shown that most colorectal cancers, demonstrating low methylation status in some genes such as hMLH1, do not silence corresponding gene expression. MSP may give positive results for tumors with such low levels of methylation with little or no biological significance.

Thus, quantitative measurement of methylation levels of DNA in cancer research is important (21, 22).

Table 3. Clinicopathological analysis of MLH1 methylation in colorectal cancer tissues

	MLH1M (>25%)	U(%)	P value
N=60	5(8.3)	55(91.7)	0.032
Age(y)			
<60	3(10.0)	27(90)	0.69
>60	2(7.1)	26(92.9)	
Gender			
Male	3(60)	22(40)	0.40
Female	2(40)	33(60)	
Ducke's stage			
A+B	2(5.9)	32(94.3)	0.34
C+D	3(13)	20(78)	
Differentiation			
Well	3(9.1)	30(91)	0.75
Moderately	1(4.5)	21(95.5)	
Poorly	0	2(100)	
Location			
Proximal	2(33.3)	4(66.7)	0.002
Distal	1(2.2)	44(97.8)	

MS-HRM analysis is a quantitative, sensitive, high throughput assessment of DNA methylation first described by Wojdacz *et al.* in 2007 (18). In this study, we used MS-HRM to analyze methylation status of the hMLH1 gene promoter and the IGF2 DMR region. We used dilution series with different ratios of methylated to unmethylated template (0, 25, 50, 75 and 100% methylated) to generate a standard curve to be used for quantification. Because low levels of methylation can have little or no biological significance, we only accepted samples with ≥25% methylation status as methylated sample for MLH1.

We detected hMLH1 methylation in 5 of 60 (8.3%) CRC tissue samples. The methylation status of hMLH1 gene promoters in CRC has been extensively studied and percentage of aberrant methylation varies between 13.2% and 53.4% in different studies (15, 23, 24). In this study, methylation pattern in CpG sites of hMLH1 gene promoter region was defined by bisulfate sequencing which showed full methylation pattern.

Table 4. Clinicopathological analysis of IGF2 hypomethylation in colorectal cancer tissues

	IGF2 hypomethylation (<50%)	50%M	P value
N=60	32 (53.3)	28(46.7)	**0.0001**
Age(y)			
<60	12(40)	18(60)	0.06
>60	18(64.3)	10(35.7)	
Gender			
Male	13(52)	12(48)	0.94
Female	18(51.4)	17(48.6)	
Ducke's stage			
A+B	20(58.8)	14(41.2)	0.41
C+D	11(47.8)	12(52.2)	
Differentiation			
Well	19(57.6)	14(42.4)	0.38
Moderately	11(50)	11(50)	
Poorly	2(100)	0	
Location			
Proximal	1(16.7)	5(83.3)	**0.026**
Distal	29(64.4)	16(35.6)	

Miyakura *et al.* showed that full methylation of the hMLH1 promoter region plays a crucial role in hMLH1 gene inactivation and carcinogenesis of MSI-H tumors in the proximal colon (25). We also analyzed any association between methylation status of hMLH1 gene and clinicopathological features in CRC patients. We found no statically significant correlation between hMLH1 methylation and any of the clinical features except an association with tumor location (p=0.002) which is similar to previous reports that showed methylation of hMLH1 is more often seen in proximal rather than distal tumors (15, 26).

Demethylation can also target specific loci in carcinogenesis and IGF2 DMR is one such region. In cancer, demethylation may be passively mediated by failure to maintain

methylation during replication or can be actived via DNA repair mechanisms (27). It has been previously reported that DNA methylation changes at the IGF2/H19 locus is related to age(28) and it has been indicated that methylation levels at this locus may be determined by heritable factors (29).

In the present study, IGF2 DMR hypomethylation was found in 32 of 60 (53.3%) of tumors and 5% of normal adjacent tissues. We showed that IGF2 DMR hypomethylation correlated with tumor location and a tendency of association with age (p=0.026 and p=0.06 respectively) which is in line with a previous report showing strong correlation between age and hypomethylation (9).

Conclusion

Our study showed that IGF2 DMR hypomethylation occurs more often after 60 years of age. As previously suggested, genetic factors predispose to hypomethylation but environmental or age-related factors likely increase hypomethylation (9). Since IGF2 DMR hypomethylation is prevalent in colorectal cancer tissue in contrast with its adjacent normal tissues, IGF2 DMR hypomethylation can be a diagnostic indicator of colorectal cancer and can be used as a suitable marker for cancer screening along with other markers before use of cancer screening (30-32). Due to the limited number of patients, further studies will be necessary to confirm these findings.

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Conflict of Interest

The authors declare no conflict of interest.

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