Bioengineered

ISSN: 2165-5979 (Print) 2165-5987 (Online) Journal homepage: https://www.tandfonline.com/loi/kbie20

Aberrant methylated EDNRB can act as a potential diagnostic biomarker in sporadic colorectal cancer while KISS1 is controversial

Reza Mousavi Ardehaie, Shahriar Hashemzadeh, Shahin Behrouz Sharif, Morteza Ghojazadeh, Ladan Teimoori-Toolabi & Ebrahim Sakhinia

To cite this article: Reza Mousavi Ardehaie, Shahriar Hashemzadeh, Shahin Behrouz Sharif, Morteza Ghojazadeh, Ladan Teimoori-Toolabi & Ebrahim Sakhinia (2017) Aberrant methylated EDNRB can act as a potential diagnostic biomarker in sporadic colorectal cancer while KISS1 is controversial, Bioengineered, 8:5, 555-564, DOI: 10.1080/21655979.2017.1283458

To link to this article: https://doi.org/10.1080/21655979.2017.1283458

Accepted author version posted online: 31 Jan 2017.
Published online: 23 Feb 2017.

BIOENGINEERED
2017, VOL. 8, NO. 5, 555–564
https://doi.org/10.1080/21655979.2017.1283458

Taylor & Francis
Taylor & Francis Group

RESEARCH PAPER

Aberrant methylated EDNRB can act as a potential diagnostic biomarker in sporadic colorectal cancer while KISS1 is controversial

Reza Mousavi Ardehaie[a,e], Shahriar Hashemzadeh[b,c], Shahin Behrouz Sharif[a,e], Morteza Ghojazadeh[d], Ladan Teimoori-Toolabi[e], and Ebrahim Sakhinia[a]

[a]Department of Biochemistry and Clinical Laboratory, Division of Medical Genetics, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran; [b]Department of General & Vascular Surgery, Tabriz University of Medical Sciences, Tabriz, Iran; [c]Tuberculosis and lung research center, Tabriz University of Medical Sciences, Tabriz, Iran; [d]Liver and Gastrointestinal Disease Research Center, Tabriz University of Medical Sciences, Tabriz, Iran; [e]Molecular Medicine Department, Pasteur Institute of Iran, Tehran, Iran

ABSTRACT

Cancers are among the most serious threats of human health worldwide. Survival and mortality rates of colorectal cancer (CRC) strongly depend on the early diagnosis. The aberrant methylation pattern of genes as a diagnostic biomarker can serve as a practical option for timely detection and contribute subsequently to the enhancement of survival rate in CRC patients, since methylation changes are not only frequent but also can occur in initial tumorogenesis stages. It has been indicated that EDNRB and KISS1 genes are hypermethylated through progression and development of CRC. In current study, after extraction of genomic DNA from 45 paired tumor and adjacent non-cancerous tissue samples and treatment with bisulfite conversion, the methylation status of EDNRB and KISS1 CpG rich regions were assessed quantitatively using MS-HRM assay to determine practicability of these aberrant methylations for diagnosis of sporadic CRC and its discrimination from corresponding normal tissues. The results showed that the methylation distribution differences, comparing tumor tissues with their adjacent non-cancerous tissues, were statistically significant in all selected locations within EDNRB gene promoter ($P < 0.001$); they had also some correlations with tumor stage and grade. Nonetheless, methylation distribution in KISS1 gene CpG rich region revealed no statistically significant differences between CRC and adjacent non-cancerous tissues ($P = 0.060$). Overall, it can be concluded that aberrant methylated EDNRB can be a promising potential diagnostic biomarker for CRC, while KISS1 is controversial and needs to be more investigated.

ARTICLE HISTORY
Received 31 October 2016
Revised 8 January 2017
Accepted 12 January 2017

KEYWORDS
biomarker; colorectal cancer; EDNRB; hypermethylation; KISS1; MS-HRM assay

Introduction

Colorectal cancer (CRC) is a common gastrointestinal malignancy with different incidence and mortality in various populations, however, it is generally the third most prevalent cancer and the fourth most common cause of cancer-related deaths worldwide.[1,2] Colorectal cancer genetically occurs in 2 different forms including familial (hereditary) and sporadic (non-hereditary) but the later cases are often more common.[3,4] Colorectal cancer is usually identified in late stages with occasional metastasis and invasion to adjacent organs since its clinical symptoms are almost silent in early stages.[2,5] The survival and mortality of CRC patients strongly depend on the time of diagnosis.[6] Diagnosis of CRC during initial stages before the occurrence of metastasis could actually rise survival rate and lower the number of deaths caused by CRC.[6] Therefore, early diagnosis of CRC, especially sporadic cases due to their higher frequency and absence of valuable prognostic information, can play an essential role in treatment and improvement of CRC patients.

In colorectal cancer cells, DNA methylation is the most significant event of epigenetic modifications and frequently happens with other genetic alterations during the transformation of normal epithelial cells and clinically tumor tissue progression from early stages to late stages.[7] Indeed, DNA methylation is not the merely cause of cancers but has significant pathological effects and driver roles in the cancer development.[7] This methylation process occurs mainly on cytosine

CONTACT Ladan Teimoori-Toolabi lteimoori@pasteur.ac.ir Molecular Medicine Department, Pasteur Institute of Iran, Tehran, Iran; Ebrahim Sakhinia esakhinia@yahoo.co.uk Department of Biochemistry and Clinical Laboratories, Division of Medical Genetics, Faculty of Medicine, Connective Tissue Disease Research Center, Tabriz Genetic Analysis Center (TGAC), Tabriz University of Medical Sciences, Tabriz, Iran.

bases in CpG-rich sequences of DNA, which are mostly located within promoter regions and less commonly in intron and exon sequences of genes.[8] Methylation and/or hypermethylation of these regions, which are known as CpG islands, catalyzed by DNA methyltransferases (DNMTs) and S-Adenosyl methionine (SAM) as a donor of methyl group, leads to repression or reduction of target gene expression in transcriptional level and subsequently changes normal cellular functions.[9] Principally, the genes containing CpG islands, which involve in cell growth, tumor suppression, carcinogenesis, invasion, and metastasis, undergo hypermethylation in tumor cells.[10,11] Recently, it has been suggested that the frequencies of DNA methylation occurrence are much more than genetic alterations in colorectal cancer.[12] Thus, these can be appropriate molecular biomarkers for early detection and stage diagnosis of colorectal cancer. For this reason, several studies have been performed and some of them non-quantitatively showed that EDNRB and KISS1 genes are subjected to methylation alterations in colorectal cancer.[13-15]

Endothelin receptor type B (EDNRB) gene, which is mapped to chromosome region 13q22, encodes a receptor which is a member of the G-protein coupled receptor superfamily and involves normally in the development of embryonic and enteric ganglia.[13,16,17] The interaction of this receptor with endothelins leads to the releasing of $InsP_3$ and Ca^{2+} as second messengers.[18] It is suggested that EDNRB gene is one of the important causes of Hirschsprung's disease (HSCR).[17] In the tumor development, transcript expression of this gene is decreased through hypermethylation of CpG-rich promoter regions resulting in alteration of ET1 signaling pathway,[19] which leads into proliferation, angiogenesis, and metastasis of tumors.[20-22] EDNRB gene fundamentally contains a CpG rich region that approximately starts from 5′ upstream of translation start site to a short while after that, as it is a potential region for transcriptional regulation of gene expression by epigenetic mechanisms in CRC.[13]

KISS1 gene maps to chromosome 1q32 and produces kisspeptin family.[23] These proteins at different sizes have originated from the cleavage of a premature 145 amino acids peptide that is encoded by KISS1 gene.[24] Normally, kisspeptins regulate the invasive properties of trophoblast during placenta formation and puberty onset by means of binding to kisspeptins receptor, which is known as KISS1-derived peptide receptor (KISS1R) and is a member of G-protein coupled receptor family (GPCR).[25] It is demonstrated that KISS1 expression is low or reduced in transcriptional level in bladder cancer and CRC, which may be influenced by hypermethylation of CpG rich regions in 5′ upstream and 3′ downstream of KISS1 gene.[14,15,26] It is believed that kisspeptins serve as metastasis suppressors in some cancers,[25] however, this inhibition function is not clearly understood. Nonetheless, KISS1R activation through kisspeptins may lead to suppression of ERK activation, which culminates with a reduction in matrix metalloproteinase 9 (MMP-9) expression and ultimately inhibition of tumor metastasis.[24,27]

Regarding the aforementioned points, analysis of DNA methylation in EDNRB and KISS1 genes seems to be important in developing new diagnostic biomarkers; therefore, this study aims at quantitatively analyzing the methylation status of these genes in sporadic CRC patients using Methylation-Sensitive High Resolution Melting (MS-HRM) assay. Furthermore, the correlation of clinicopathological parameters and aberrant methylation of both genes will be detected.

Results

The methyaltion status of EDNRB and KISS1 in CRC and adjacent non-cancerous colonic tissues

The evaluation of DNA methylation status was performed using a series of standard methylated samples ranged from completely methylated to entirely unmethylated (100%, 75%, 50%, 25% and 0% methylated), as control DNA samples of the MS-HRM assay. The normalized melting curves of analyzed locations for control samples are shown in Fig. 1. Four locations of EDNRB and one location of KISS1 were selected to assess their methylation status in 45 paired specimens. Methylation of EDNRB were frequently detected in tumor tissue samples than in adjacent non-cancerous tissue samples in all locations ($P < 0.001$), in which the median methylation values of locations 1, 2, 3 and 4 were 75% (range 12.5 to 100%), 75% (range 12.5 to 100%), 75% (range 25 to 100%) and 62.5% (range 12.5 to 100%) respectively, whereas in matched adjacent non-cancerous tissues were 12.5% (range 0 to 75%), 25% (range 0 to 62.5%), 37.5% (range 0 to 75%) and 25% (range 0 to 75%), respectively (Table 2, Fig. 2A and Fig. 3A). Unexpectedly, the results showed that methylation status differences were not statistically significant in the chosen

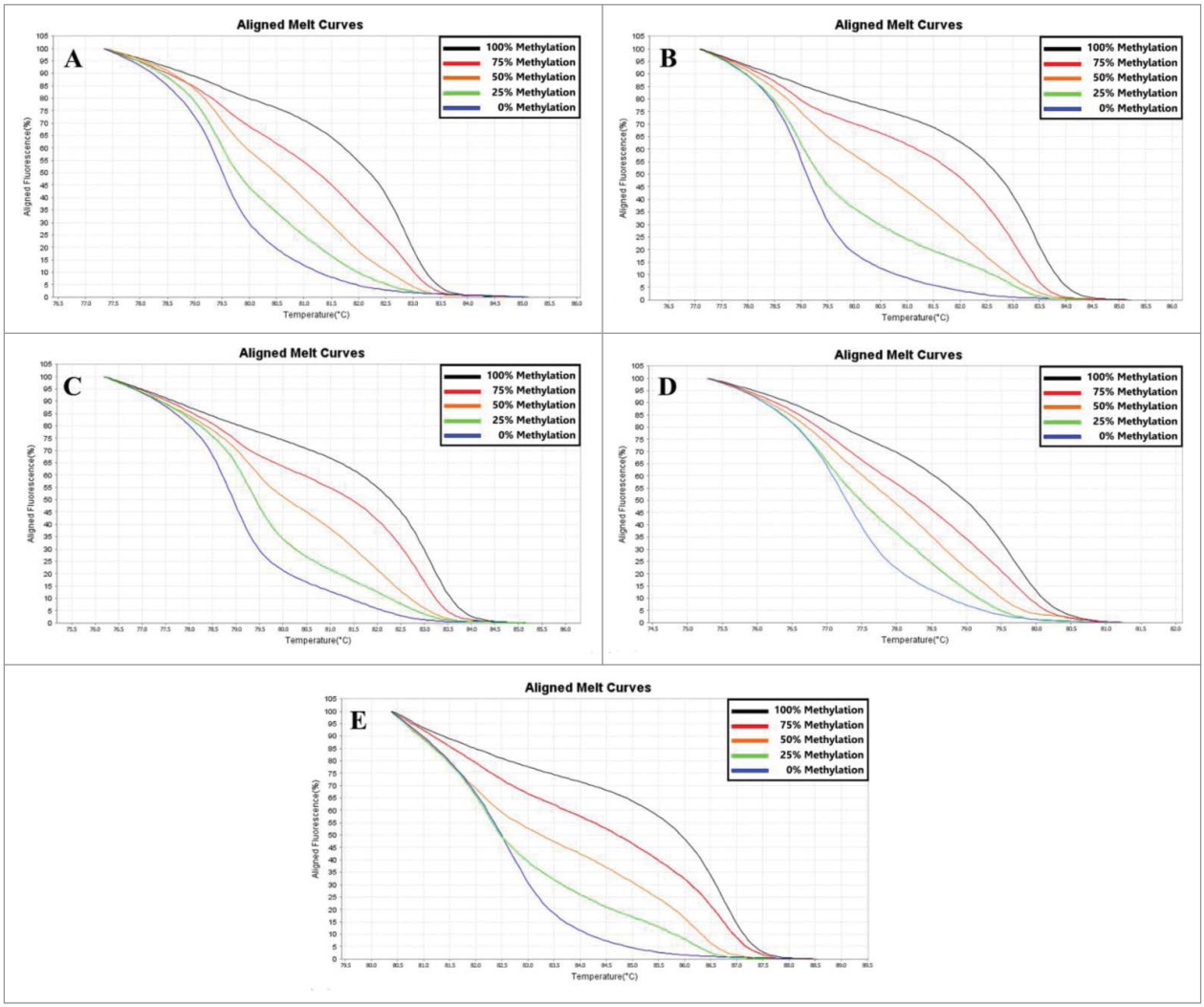

Figure 1. High Resolution Melting (HRM) aligned melt curves for 5 analyzed locations. (A) Indicates control curves for EDNRB gene 1st location. (B) Indicates control curves for EDNRB gene 2st location. (C) Indicates control curves for EDNRB gene 3st location. (D) Indicates control curves for EDNRB gene 4st location. (E) Indicates control curves for KISS1 gene selected location. In all 5 figures, blue lines indicate 0% control, green lines indicate 25% control, orange lines indicate 50% control, red lines indicate 75% control and the black lines indicate 100% controls.

location of KISS1 ($P = 0.060$). Methylation level of KISS1 in the tumor tissues (median value, 12.5% and range 0 to 12.5%) compared with their adjacent non-cancerous tissues (median value, 0% and rang 0 to 12.5%) indicates the existence of low level methylation in examined location of KISS1 (Table 2, Fig. 2B and Fig. 3B).

Differences in aberrant methylation pattern of selected locations of EDNRB

Interestingly, the methylation distribution of the studied locations of EDNRB gene in both tumor and adjacent normal tissues was relatively different. The mean methylation percentage of locations 1, 2, 3 and 4 were 71.40, 67.50, 67.50 and 64.44, whereas, in the adjacent non-cancerous tissues were 22.77, 26.94, 36.38 and 23.88%, respectively (Fig. 3A). The highest difference between the mean methylation percentage of tumor and adjacent non-cancerous tissue was observed in location 1 (the farthest location from the transcriptional start site, ATG codon).

Relationships of EDNRB and KISS1 methylation status with clinicopathological features in CRC

The analytic results of the correlation between clinicopathological characteristics and methylation levels of

Table 1. The information of primers used for MS-HRM assay.

Target sequence	Sequence (5′–3′)	T_a* (C°)	Amplicon size (bp)	Screened CpGs**	Amplified region***
EDNRB gene (Location 1)	F: AGGTTGGGTAAAGGAAGGAG R: CGCTACTCCCTAACTAACTAAACT	59	160	11	77919267–77919107
EDNRB gene (Location 2)	F: GTTGAGAGGGTATTAGGAAGGAGT R:CGCAATTTCAAAACCTAAAAACAAAC	59	211	14	77918821–77918610
EDNRB gene (Location 3)	F: CGGGTAGTTGATTTAAGTGTTTTGT R:CCTCTCTCCTCTCCCCAAATC	59	189	12	77918673–77918484
EDNRB gene (Location 4)	F: GATTTGGGGAGAGGAGAGAGG R: CGTCCTATCTCCTTTAAACACCTC	59	163	9	77918505–77918342
KISS1 gene	F: CGAAGGAGTTTTAGTTGTAGTT R: CCAACTACTACTACCAAACTAAAC	58	181	14	204190548–204190729

*Appropriate annealing temperature **Number of CpGs included in the amplicon ***Nucleotide numbers.

EDNRB gene in 4 analyzed locations showed that the aberrant methylation pattern was independent of the age, gender, tumor location and size and smoking status ($P > 0.05$). However, methylation levels of location 1 and location 4 of EDNRB gene were significantly correlated with tumor grade ($P = 0.008$ and $P = 0.033$, respectively), moreover, the location 1 was also in correlation with tumor stage ($P = 0.035$).

On the other hand, the methylation percentage of studied location for KISS1 gene was relatively low in all samples, significant correlation was not found between methylation status and any of clinicopathological characteristics including age, gender, tumor features and smoking status ($P > 0.05$). Detailed information for these correlations are shown in Table 3.

Sensitivity and specificity of methylated EDNRB and KISS1 genes

The sensitivity and specificity of tests were obtained using receiver operating characteristic (ROC) curves analysis to determine the capability of EDNRB and KISS1 methylations for CRC diagnosis and discrimination between tumor and adjacent normal tumor-free tissue (Fig. 4). Additionally, positive predictive value (PPV), negative predictive value (NPV), and the accuracy were evaluated for detection of CRC by MS-HRM assay (Table 4). Although, all of the assessed locations in EDNRB gene promoter showed promising sensitivity and specificity values, it can be derived from Fig. 4 and Table 4 that when the cutoff point is 43.75 percent, the methylation status in first location of EDNRB gene can be a hopeful diagnostic biomarker with slightly higher sensitivity, specificity, PPV, NPV and accuracy in comparison with other locations of EDNRB gene ($P < 0.001$). In contrast, the sensitivity, specificity, PPV, NPV and accuracy of KISS1 gene methylation test were 57.8, 71.1, 66.7, 62.7 and 64.4, respectively (cutoff value, 6.25%), which were not statistically significant ($P = 0.060$).

Table 2. The *EDNRB* and *KISS1* genes methylation status in samples.

Gene name	Mean (Adjacent/Tumor)	Std. Deviation (Adjacent/Tumor)	*P*-value
EDNRB			
Location 1	22.7778/71.4000	17.5342/20.23206	<0.001
Location 2	26.9444/67.5000	16.6334/18.54050	<0.001
Location 3	36.3889/67.5000	18.6211/20.19085	<0.001
Location 4	23.8889/64.4440	21.7850/22.91839	<0.001
KISS1	3.6111/7.2222	5.72960/6.24368	0.060

Discussion

The epigenetic alterations may be involved in development and progression of cancers. CpG island hypermethylation of promoter regions of tumor suppressor genes, which also has a significant role in tumor metastasis, is a mechanism that frequently silences gene expression in transcriptional level during tumorgenesis.[28,29] Thus, aberrant DNA methylation pattern analysis can be a valuable biomarker in early diagnosis of cancers.[30,31]

It has been reported that EDNRB and KISS1 as the tumor suppressor and tumor metastasis suppressor genes, respectively, are silenced in various cancers such as colorectal cancer, which is caused by specific CpG islands' hypermethylation.[13-15] Regarding EDNRB, which encodes a G-protein coupled receptor, it is implicated that hypermethylation of CpG dinucleotides in 5′ flanking region happens during CRC progression.[13] Indeed, this aberrant methylation is associated with the EDNRB downregulation and silencing that may be involved in the cell proliferation,

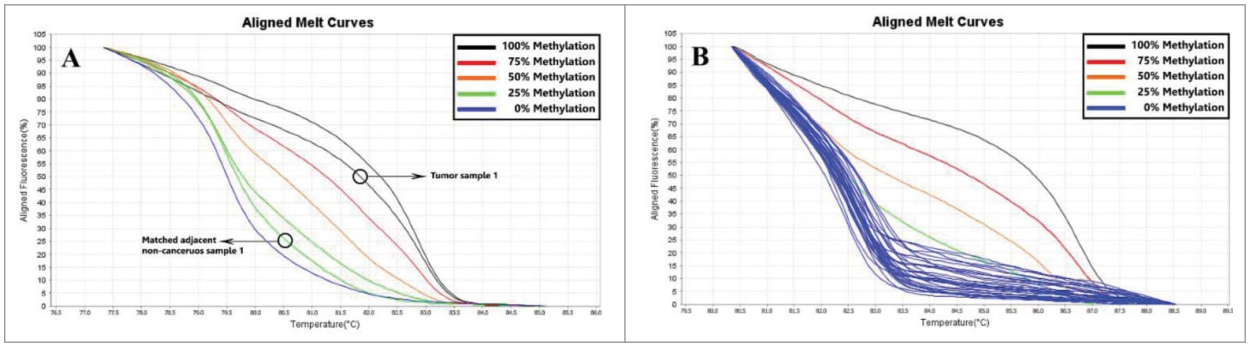

Figure 2. Represents curves for samples and controls. (A) Indicates tumor and adjacent non-cancerous samples exhibiting different methylation ratios in the 1st analyzed location of EDNRB gene (0–25% for matched adjacent non-cancerous sample, 75–100% for tumor sample). (B) Indicates nearly half of the analyzed samples for KISS1, which shows them to be almost under 25% methylation ratio.

angiogenesis and metastasis of several tumors.[20-22] In a similar way, hypermethylation of 5′ and 3′ flanking regions of KISS1 gene, which encodes metastin proteins, culminates with an expression decrease in transcriptional level that likely results in certain physiologic changes in affected cells during cancer progression.[14,15]

In the present study, EDNRB hypermethylation in CRC confirmed quantitatively for the first time by MS-HRM assay, concordant with what previously found in Chen et al study.[13] Methylation specific PCR (MSP) results in their study, which is a non-quantitative technique, showed that EDNRB hypermethylation frequency in CRC tumor tissues was higher than adjacent normal samples (92.86 versus 59.52, $P = 0.001$) and this aberrant methylation was correlated with EDNRB downregulation. Nevertheless, they found no significant association between methylation distribution in the EDNRB gene promoter and clinicopathological characteristics of tumors. While the current study, with higher sensitivity and accuracy in evaluation of methylation status using MS-HRM,[32] was able to identify the significant correlation of EDNRB promoter methylation status with tumor stage as well as tumor grade. Moreover, there are some reports that methylation levels of EDNRB promoter were related with tumor invasion and pathological stage in gastric and prostate cancers, respectively.[33,34]

Moya et al, using methylation specific PCR (MSP) and bisulfite sequencing PCR (BSP), revealed that the 3′ flanking region of KISS1 was extra methylated in CRC tumor specimens comparing to corresponding normal tissues. Likewise, the poor clinical outcome and advanced CRC tumor stages were indicated when this region was hypermethylated.[14] Moreover, Chen et al declared that there is hypermethylation of this

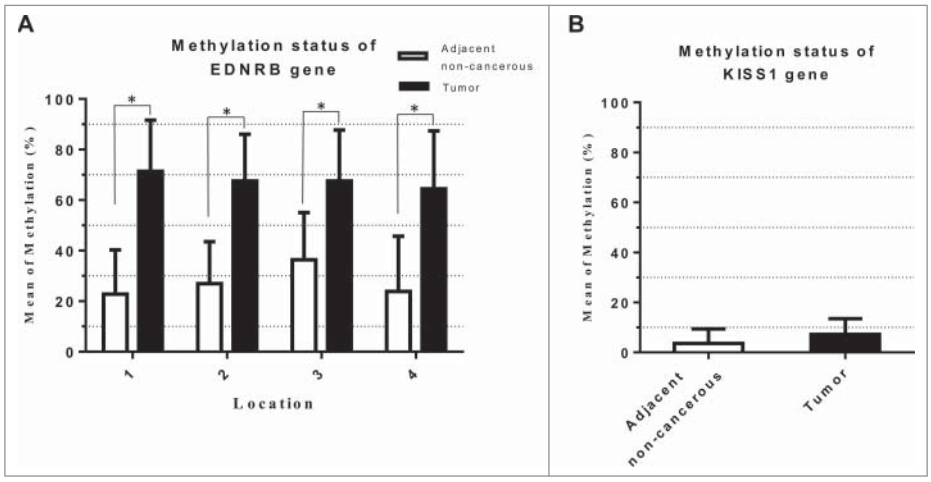

Figure 3. Mean of methylation status percentage of EDNRB and KISS1 genes. Data are expressed as mean ± SD from 3 independent experiments ($^*P < 0.001$).

Table 3. The correlations of cilinicopathological characteristics of *EDNRB* and *KISS1* gene methylation status.

		EDNRB methylation				
Characteristics (n = 45)	number	(Location 1)***P*-value**	(Location 2) ***P*-value**	(Location 3) ***P*-value**	(Location 4) ***P*-value**	KISS1 methylation *P*-value
Age		0.135	0.205	0.307	0.131	0.205
<50	8					
≥ 50	37					
Gender		0.539	0.811	0.494	0.293	0.537
Male	19					
Female	26					
Tumor Location		0.654	0.954	0.444	0.800	0.081
Cecal	3					
Right colon	12					
Transverse colon	4					
Left colon	5					
Sigmoid colon	13					
Rectosigmoid	8					
Tumor Size		0.674	0.953	0.797	0.982	0.264
<5	24					
≥ 5	21					
Tumor Grade		0.008	0.059	0.079	0.033	0.950
G1	20					
G2	23					
G3	2					
Tumor Stage		0.035	0.068	0.315	0.511	0.672
I	8					
II	17					
III	14					
IV	6					
Smoking		0.079	0.204	0.054	0.091	0.431
No	38					
Yes	7					

gene in 83.33% of primary CRC vs. 6.30% in normal intestine specimens and also there is an association with the depth of local invasion, tumor differentiation and tumor metastatic features.[15] In contrast, it is interesting that the present study using MS-HRM assay discovered no statistically significant differences between 3′ flanking region methylation of tumor samples and adjacent tumor-free samples for KISS1;

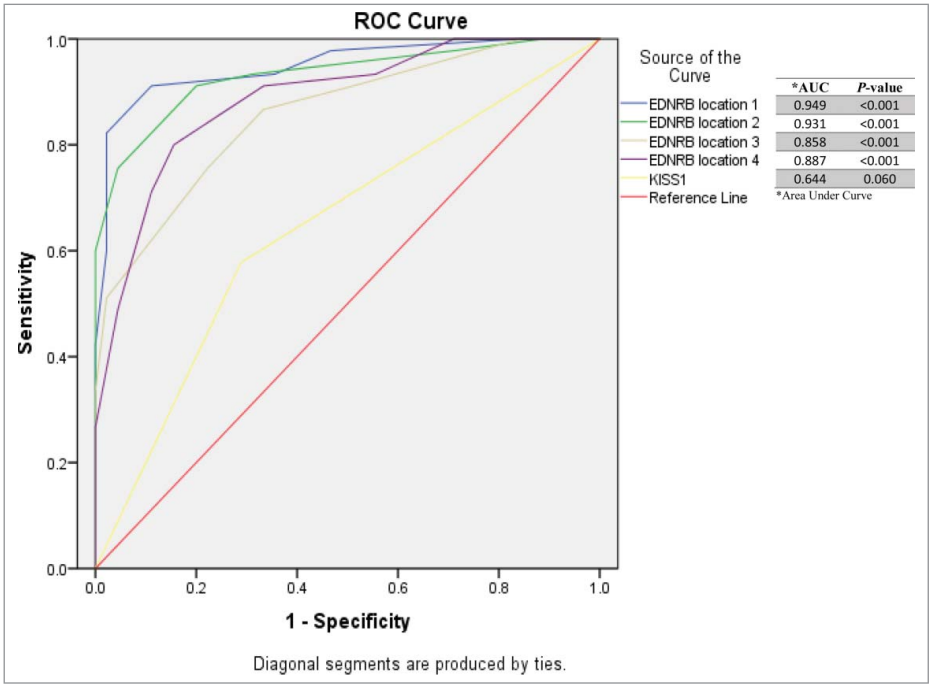

Figure 4. Receiver Operating Characteristic (ROC) curve analysis of EDNRB and KISS1 methylation using MS-HRM assay for discriminating between CRC and adjacent non-cancerous samples.

Table 4. Diagnostic performance of *EDNRB* and *KISS1* MS-HRM.

Gene name	AUC* (95% CI)	Cutoff value (%)**	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
EDNRB							
Location 1	0.949 (0.904–0.994)	43.75	91.1	88.9	89.1	90.9	90.0
Location 2	0.931 (0.878–0.984)	43.75	91.1	80.0	82.0	90.0	85.6
Location 3	0.858 (0.783–0.934)	56.25	75.6	77.8	77.2	76.1	76.7
Location 4	0.887 (0.819–0.954)	43.75	80.0	84.4	83.7	80.8	82.2
KISS1							
	0.644 (0.530–0.759)	6.25	57.8	71.1	66.7	62.7	64.4

*Area Under Curve, **Percentage of methylation.

additionally, there was no relationship with the clinicopathological characteristics of tumor samples and methylation status. Therefore, this study could not validate their assumptions despite using MS-HRM assay as a quantitative method that can rapidly identify DNA methylation levels with higher sensitivity and specificity.[32] It has been claimed that capability of this method for detecting methylation levels is very similar to those determined by the gold standard pyrosequencing in sensitivity and specificity due to total scanning of individual CpGs within the target sequence, but it cannot separately detect methylation levels of any CpGs as pyrosequencing method.[35] Indeed, the detectability of this method has approximately no false positive results, which has been seen considerably in MSP that has lower sensitivity and specificity comparing to MS-HRM.[32] Then, in this study, the discrepancy in results of KISS1 methylation status with previous studies may have been resulted from the different application of techniques (quantitative vs. qualitative). Moreover, only the 3′ flanking region of KISS1 was surveyed in current study, while it has been introduced that its 5′ flanking region also undergoes methylation alterations.[15] Therefore, the difference between selected CpG containing regions could be another reason for contradiction of the results. As a recommendation, both 2 regions should be assessed simultaneously to probably address this confliction.

Although it has been suggested that KISS1 gene expression levels is reduced in various cancers,[14] which has unfortunately not been assayed herein, Kostakis et al proposed that the KISS1 expression downregulation could be an early event outcome when colonic cells transform toward malignancy, yet the KISS1 expression is being upregulated persistently during tumor progression, especially during expansion in size and invasive properties.[25] Hence, considering the reports indicating decrease of KISS1 gene expression in various cancers, it seems possible that this reduction also could be resulted from the other key mechanisms involved in modulation of gene expression such as chromatin remodeling, post-transcriptional and post-translational modifications in addition to the gene-specific methylation which is not observed in KISS1 in present study. Moreover, it has been demonstrated that miR199b expression, which negatively regulates expression of STRT1 gene, is reduced in CRC. The initiation of STRT1 upregulation leads to increased deacetylation of CREB transcription factor and its inactivation, which ultimately causes inhibition of KISS1 gene expression, since it is one of potential CREB targets.[36] Furthermore, it has also been indicated that suppression of KISS1 gene expression can result from expression silencing of TCF21 transcription factor with increased miR-21 in renal cell carcinoma.[37] Therefore, considering the complexity of gene expression regulation, more investigations might be needed to be done in this area.

Another point contributing to surprising results for KISS1 methylation may be due to the importance of selecting the region containing CpG islands. The present study revealed that the mean of methylation levels in distinct selected locations of EDNRB promoter region were different from each other. Actually, the mean methylation levels of the first location displayed the highest difference between tumor and adjacent non-cancerous tissue and also had the highest sensitivity and specificity in CRC diagnosis as well as a higher accuracy. This issue likely implicates the important role of CpG methylation in this location of EDNRB for transcription modulation, which is not considered in current study that different distribution of methylation how much affects regulation of gene expression and whether the CpG islands methylation levels in 5′-flanking region of EDNRB, which is distal part from the translation start site, has more determinant role in controlling gene transcription. Indeed, it

seems that selection of CpG containing region should be considered for methylation status assessment with respect to differences observed in methylation levels of different locations.

In conclusion, the current study, using MS-HRM assay, has demonstrated that the 5′ flanking region of EDNRB gene is hypermethylated in human CRC and it can be a potential diagnostic biomarker for CRC discrimination from adjacent normal non-cancerous tissues. The combination of locations 1 and 2, especially, can be used as a powerful biomarker, in which the first location is significantly correlated with tumor stage and grade, and the second one is aberrantly methylated independent of any clinicopathological features. However, the results for the chosen region of KISS1 gene was unexpected and more investigations must be performed to resolve the discrepancy of the findings in this study and previous ones.

Materials and methods

Tissue specimens and patients

In the present study, which was approved by Connective Tissue Disease Research Center of Tabriz University of Medical Science with cooperation of Pasteur Institute of Tehran, a total number of 45 paired sporadic colorectal cancer (CRC) and adjacent non-cancerous colonic tissue samples were obtained from CRC patients who underwent surgical operation (median age 59, age range 29–83) at Imam Reza and Amiralmomenin Hospitals, Tabriz, Iran during September 2014 to July 2015. None of the patients had undergone chemotherapy or radiotherapy treatments before surgery. After resection, both tumor and matched adjacent non-cancerous tissue samples have been histologically and pathologically confirmed by consultant pathologist and immediately stored in −80 C° until genomic DNA extraction. The clinicopathological data including age, gender, CRC tumor features (anatomic location, size, grade and stage of tumor) and smoking status were collected from medical records and patients' history.

Genomic DNA isolation and sodium bisulfite modification

Genomic DNA extraction was performed using CinnaPure-DNA kit (Cinna gene, Iran) according to the manufacturer's protocol. After extraction, DNA concentrations were measured by NonoDrop spectrophotometer; Samples with the final concentration of $>$ 100 ng/μl and A260/A280 ratio in the range of 1.7–1.9 were acceptable. In the next step, extracted DNA samples were subjected to sodium bisulfite conversion using EZ DNA Methylation-Gold Kit (Zymoresearch, CA), based on the instructions provided by the manufacturer. Finally, the converted DNA (bis-DNA) was stored at −20°C until MS-HRM assay.

Methylation-sensitive high resolution melting (MS-HRM) assay

The converted DNA was applied to amplify CpG rich regions which were predicted using MethPrimer online software, these regions were located in the upstream of EDNRB gene and downstream of KISS1 gene. For this purpose, PCR amplification was performed on Step one plus Real-time PCR (ABI applied biosystems, USA) using sets of specific primers that are listed in Table 1. The final volume of each reaction was 20 μl, including 2 μl of converted template DNA, 10 μl of master mix (SYBR Premix Ex Taq II, TAKARA, Japan), 1.2 ul of primer pair (5 pmol) and 6.8 μl of double distilled water. The condition of PCR amplification was followed in: holding step at 95 °C for 30 s and followed by 40 cycles of denaturation at 95 °C for 5 s, optimal annealing temperatures (T_A) of each primer pairs (Table 1) for 15 s and extension at 72 °C for 15 s. As soon as it was finished, HRM step with the continuous ramp rate of the acquisition of 0.3°C per 15 sec was sequentially initiated in a rising temperature from 60°C to 95°C. The control DNA (HUM Diagnostic Biotechnology, Iran) was included as DNA samples with known methylation ratios for standard curve series (0%, 25%, 50%, 75% and 100%) in each assay for deduction of the methylation status of unknown samples. The melting curves of all PCR products were run on HRM software v.2.2 (ABI applied biosystems, USA). With respect to normalization of melting curves relative to 2 normalization regions before and after the major decrease of fluorescence, the output fluorescence plots from each reaction were displayed relative to the percentage of unknown samples' methylation status comparing to the standard curves'.

Statistical analysis

The statistical analyses were performed using non-parametric tests of IBM SPSS version 19, as data

were not normally distributed. The U Mann-Whitney test was used to examine differences in the DNA methylation status between the tumor and adjacent non-cancerous colonic tissue specimens. The association of EDNRB and KISS1 genes methylation status between tumor tissues, age, gender, tumor size, and smoking and moreover between tumor tissues, location, grade, and stage of tumor was respectively analyzed by the U Mann-Whitney and Kruskal-Wallis tests. The receiver operating characteristic (ROC) Curve analysis was applied to evaluate sensitivity and specificity of methylation status for the analyzed regions. In this study, P-value < 0.05 was assumed statistically significant.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Acknowledgments

The authors would like to express their sincerely appreciation to all patients who participated in this study.

References

[1] Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer 2015; 136:E359-86; PMID:25220842; https://doi.org/10.1002/ijc.29210

[2] Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer 2010; 127:2893-917; PMID:21351269; https://doi.org/10.1002/ijc.25516

[3] Mendelsohn J, Howley PM, Israel MA, Gray JW, Thompson CB. The molecular basis of cancer. Elsevier Health Sciences 2014.

[4] Jasperson KW, Tuohy TM, Neklason DW, Burt RW. Hereditary and familial colon cancer. Gastroenterol 2010; 138:2044-58; https://doi.org/10.1053/j.gastro.2010.01.054

[5] Center M, Siegel R, Jemal A. Global cancer facts & figures. Atlanta: American Cancer Society 2011:1-52.

[6] O'Connell JB, Maggard MA, Ko CY. Colon cancer survival rates with the new American joint committee on cancer sixth edition staging. J Natl Cancer Inst 2004; 96:1420-5; PMID:15467030; https://doi.org/10.1093/jnci/djh275

[7] Lao VV, Grady WM. Epigenetics and colorectal cancer. Nat Rev Gastroenterol Hepatol 2011; 8:686-700; PMID:22009203; https://doi.org/10.1038/nrgastro.2011.173

[8] Olkhov Mitsel E, Bapat B. Strategies for discovery and validation of methylated and hydroxymethylated DNA biomarkers. Cancer Med 2012; 1:237-60; PMID:23342273; https://doi.org/10.1002/cam4.22

[9] Lewandowska J, Bartoszek A. DNA methylation in cancer development, diagnosis and therapy - Multiple opportunities for genotoxic agents to act as methylome disruptors or remediators. Mutagenesis 2011; 26:475-87; PMID:21551264; https://doi.org/10.1093/mutage/ger019

[10] van Engeland M, Derks S, Smits KM, Meijer GA, Herman JG. Colorectal cancer epigenetics: Complex simplicity. J Clin Oncol 2011; 29:1382-91; PMID:21220596; https://doi.org/10.1200/JCO.2010.28.2319

[11] Sjöblom T, Jones S, Wood LD, Parsons DW, Lin J, Barber TD, Mandelker D, Leary RJ, Ptak J, Silliman N, et al. The consensus coding sequences of human breast and colorectal cancers. Science 2006; 314:268-74; PMID:16959974; https://doi.org/10.1126/science.1133427

[12] Schuebel KE, Chen W, Cope L, Glöckner SC, Suzuki H, Yi JM, Chan TA, Van Neste L, Van Criekinge W, van den Bosch S, et al. Comparing the DNA hypermethylome with gene mutations in human colorectal cancer. PLoS Genet 2007; 3:e157; https://doi.org/10.1371/journal.pgen.0030157

[13] Chen C, Wang L, Liao Q, Huang Y, Ye H, Chen F, Xu L, Ye M, Duan S. Hypermethylation of EDNRB promoter contributes to the risk of colorectal cancer. Diagn Pathol 2013; 8:1; PMID:23298385

[14] Moya P, Esteban S, Fernandez-Suarez A, Maestro M, Morente M, Sánchez-Carbayo M. KiSS-1 methylation and protein expression patterns contribute to diagnostic and prognostic assessments in tissue specimens for colorectal cancer. Tumor Biol 2013; 34:471-9; https://doi.org/10.1007/s13277-012-0572-3

[15] Chen SQ, Chen ZH, Lin SY, Dai QB, Fu LX, Chen RQ. KISS1 methylation and expression as predictors of disease progression in colorectal cancer patients. World J Gastroenterol 2014; 20:10071; PMID:25110434; https://doi.org/10.3748/wjg.v20.i29.10071

[16] Druckenbrod NR, Powers PA, Bartley CR, Walker JW, Epstein ML. Targeting of endothelin receptor−B to the neural crest. Genesis 2008; 46:396-400; PMID:18693272; https://doi.org/10.1002/dvg.20415

[17] Sánchez-Mejías A, Fernández RM, López-Alonso M, Antiñolo G, Borrego S. Contribution of RET, NTRK3 and EDN3 to the expression of Hirschsprung disease in a multiplex family. J Med Genet 2009; 46:862-4; PMID:19556619; https://doi.org/10.1136/jmg.2009.067819

[18] Schussel J, Zhou XC, Zhang Z, Pattani K, Bermudez F, Jean-Charles G, McCaffrey T, Padhya T, Phelan J, Spivakovsky S, et al. EDNRB and DCC salivary rinse hypermethylation has a similar performance as expert clinical examination in discrimination of oral cancer/dysplasia versus benign lesions. Clin Cancer Res 2013; 19:3268-75; PMID:23637120; https://doi.org/10.1158/1078-0432.CCR-12-3496

[19] Davenport A, Maguire J. Endothelin. The Vascular Endothelium I. Springer, 2006:295-329.

[20] Zhao BJ, Sun DG, Zhang M, Tan SN, Ma X. Identification of aberrant promoter methylation of EDNRB gene in esophageal squamous cell carcinoma. Dis Esophagus

2009; 22:55-61; PMID:18564167; https://doi.org/10.1111/j.1442-2050.2008.00848.x

[21] Lahav R, Heffner G, Patterson PH. An endothelin receptor B antagonist inhibits growth and induces cell death in human melanoma cells in vitro and in vivo. Proc Natl Acad Sci U S A 1999; 96:11496-500; PMID:10500205; https://doi.org/10.1073/pnas.96.20.11496

[22] Mallat A, Fouassier L, Preaux A, Gal C, Raufaste D, Rosenbaum J, Dhumeaux D, Jouneaux C, Mavier P, Lotersztajn S. Growth inhibitory properties of endothelin-1 in human hepatic myofibroblastic Ito cells. An endothelin B receptor-mediated pathway. J Clin Invest 1995; 96:42; PMID:7615814; https://doi.org/10.1172/JCI118052

[23] Sanchez-Carbayo M, Capodieci P, Cordon-Cardo C. Tumor suppressor role of KiSS-1 in bladder cancer: loss of KiSS-1 expression is associated with bladder cancer progression and clinical outcome. Am J Pathol 2003; 162:609-17; PMID:12547718; https://doi.org/10.1016/S0002-9440(10)63854-0

[24] Ji K, Ye L, Ruge F, Hargest R, Mason MD, Jiang WG. Implication of metastasis suppressor gene, Kiss-1 and its receptor Kiss-1R in colorectal cancer. BMC Cancer 2014; 14:1; PMID:24383403; https://doi.org/10.1186/1471-2407-14-723

[25] Kostakis ID, Agrogiannis G, Vaiopoulos AG, Mylona E, Patsouris E, Kouraklis G, Koutsilieris M. KISS1 expression in colorectal cancer. APMIS 2013; 121:1004-10; PMID:24033850; https://doi.org/10.1111/apm.12161

[26] Cebrian V, Fierro M, Orenes-Piñero E, Grau L, Moya P, Ecke T, Alvarez M, Gil M, Algaba F, Bellmunt J, et al. KISS1 methylation and expression as tumor stratification biomarkers and clinical outcome prognosticators for bladder cancer patients. Am J Pathol 2011; 179:540-6; PMID:; PMID:21683672; https://doi.org/10.1016/j.ajpath.2011.05.009

[27] Chen S, Chen W, Zhang X, Lin S, Chen Z. Overexpression of KiSS-1 reduces colorectal cancer cell invasion by downregulating MMP-9 via blocking PI3K/Akt/NF-κB signal pathway. Int J Oncol 2016; 48:1391-8; PMID:26847533

[28] Du YY, Dai DQ, Yang Z. Role of RECK methylation in gastric cancer and its clinical significance. World J Gastroenterol 2010; 16:904-8; PMID:20143471; https://doi.org/10.3748/wjg.v16.i8.921

[29] Rizvi MMA, Alam MS, Ali A, Mehdi SJ, Batra S, Mandal A. Aberrant promoter methylation and inactivation of PTEN gene in cervical carcinoma from Indian population. J Cancer Res Clin Oncol 2011; 137:1255-62; PMID:21698421; https://doi.org/10.1007/s00432-011-0994-0

[30] Kang GH, Lee S, Kim JS, Jung HY. Profile of aberrant CpG island methylation along the multistep pathway of gastric carcinogenesis. Lab Invest 2003; 83:635-41; PMID:12746473; https://doi.org/10.1097/01.LAB.0000067481.08984.3F

[31] Tost J. DNA methylation: An introduction to the biology and the disease-associated changes of a promising biomarker. Mol Biotechnol 2010; 44:71-81; PMID:19842073; https://doi.org/10.1007/s12033-009-9216-2

[32] Xiao Z, Li B, Wang G, Zhu W, Wang Z, Lin J, Xu A, Wang X. Validation of methylation-sensitive high-resolution melting (MS-HRM) for the detection of stool DNA methylation in colorectal neoplasms. Clin Chim Acta 2014; 431:154-63; PMID:24518356; https://doi.org/10.1016/j.cca.2014.01.044

[33] Tao K, Wu C, Wu K, Li W, Han G, Shuai X, Wang G. Quantitative analysis of promoter methylation of the EDNRB gene in gastric cancer. Med Oncol 2012; 29:107-12; PMID:21264540; https://doi.org/10.1007/s12032-010-9805-8

[34] Yegnasubramanian S, Kowalski J, Gonzalgo ML, Zahurak M, Piantadosi S, Walsh PC, Bova GS, De Marzo AM, Isaacs WB, Nelson WG. Hypermethylation of CpG islands in primary and metastatic human prostate cancer. Cancer Res 2004; 64:1975-86; PMID:15026333; https://doi.org/10.1158/0008-5472.CAN-03-3972

[35] Migheli F, Stoccoro A, Coppede F, Omar WAW, Failli A, Consolini R, Seccia M, Spisni R, Miccoli P, Mathers JC, et al. Comparison study of MS-HRM and pyrosequencing techniques for quantification of APC and CDKN2A gene methylation. PLoS One 2013; 8:e52501; PMID:23326336; https://doi.org/10.1371/journal.pone.0052501

[36] Shen ZL, Wang B, Jiang KW, Ye CX, Cheng C, Yan YC, Zhang JZ, Yang Y, Gao ZD, Ye YJ, et al. Downregulation of miR-199b is associated with distant metastasis in colorectal cancer via activation of SIRT1 and inhibition of CREB/KISS1 signaling. Oncotarget 2016; 7:35092-105; PMID:27145368

[37] Zhang H, Guo Y, Shang C, Song Y, Wu B. miR-21 downregulated TCF21 to inhibit KISS1 in renal cancer. Urology 2012; 80:1298-302. e1; PMID:23206776; https://doi.org/10.1016/j.urology.2012.08.013