Detection of aberrant methylated SEPT9 and NTRK3 genes in sporadic colorectal cancer patients as a potential diagnostic biomarker

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Abstract. Colorectal cancer (CRC) is one of the most common malignancies, and the third leading cause of cancer mortality worldwide. Timely detection of CRC in patients with earlier stages provides the highest rate of survival. Epigenetic alterations are important in the occurrence and progression of CRC, and represent the primary modifications of cancer cells. Therefore, detection of these alterations in CRC cases are thought to hold great promise as diagnostic biomarkers. It has been shown that the *SEPT9* and *NTRK3* genes are aberrantly methylated and their detection can be used as biomarkers for early diagnosis of CRC. The present study analyzed promoter methylation status of these genes in CRC patients. Genomic DNA was extracted from 45 CRC and paired adjacent healthy tissues and undergone bisulfite conversion, and the methylation status of *NTRK3* and *SEPT9* were defined using the MS-HRM assay. Our results showed that there are statistically significant differences in methylation status of *NTRK3* and specially *SEPT9* between CRC and adjacent normal tissues ($P<0.001$). High sensitivity and specificity for a specific location in *SEPT9* gene promoter as a diagnostic biomarker was observed. *SEPT9* promoter hypermethylation may serve as a promising biomarker for the detection of CRC development. However, to validate the biomarker potential of *NTRK3* there is a requirement for further investigation.

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Introduction

Colorectal cancer (CRC) is one of the most common malignancies worldwide, and the second cause of cancer related deaths in developed countries (1,2). CRC is the 3rd and the 4th commonly diagnosed cancer in Iranian men and women, respectively (3). The lack of clinical manifestations in CRC patients until the late stages of cancer is a common disease characteristic, which results in poor prognosis and high mortality. The process of carcinogenesis in primary adenomas, which are precursor lesions of colon cancer that eventually develop into colorectal carcinomas, is slow, and is a cause of the late diagnosis (4). Eighty percent of early-diagnosed patients are referred for tissue resection and are eventually cured (5). In order to reduce the morbidity and mortality of the disease, early diagnosis and treatment of CRC appears to be of critical importance, since there is a large preclinical asymptomatic stage in CRC patients (6).

There are several CRC screening methods, such as fecal occult blood testing (FOBT), barium enema, flexible sigmoidoscopy and colonoscopy. FOBT and colonoscopy are commonly used clinically; however, they have some technical restrictions and disadvantages (7). FOBT, while a simple method in practice, does not have high sensitivity and specificity. Considering that colonoscopy is the 'gold standard' method for CRC screening, its invasiveness nature and complicated required preparation procedure make patients

reluctant to choose it as an acceptable screening method (7). Therefore, developing new and useful screening methods is a high priority (8).

Sporadic CRC occurrence is due to accumulation of genetic and epigenetic changes, which cause normal epithelial cells to transform into the adenocarcinoma cells (9). There is increasing evidence that widespread epigenetic alterations are the key features of most of cancer types (10,11), and these changes may be important in the pathogenesis of CRC. Aberrant DNA methylation is one of the best known and well-defined epigenetic changes in tumors, and is a frequent mechanism for inappropriate gene silencing among tumor suppressor genes (12-14). DNA methylation is of particular interest, as it occurs in the primary stages of carcinogenesis, and hence, can be used as a marker for early detection of CRC (15).

Septin 9 (*SEPT9*) is a member of the septin gene family, which are highly conserved and encode GTP-binding proteins. Septins are multidomain proteins which together form filamentous structures that form part of cytoskeleton (16,17). Furthermore, septins belong to P-loop GTPases superclass and were first identified in yeast as key genes in cell division (18). These proteins have prominent roles in multiple cellular processes, including cell membrane rigidity, establishment of separate cellular domains by creating membrane diffusion barriers, providing scaffold for localization of proteins to certain subcellular regions and determination of cell polarity (16,18,19). The precise mechanism of *SEPT9* molecular function in colon cancer pathogenesis has not yet been clearly described (20), however, several studies suggest possible roles in different malignancies, including leukemia (21), breast and ovarian cancer (22-24), brain tumors (25) and CRC (19,26-28).

The methylation status of the *SEPT9* gene has been examined previously in CRC patients, as well as cases with precancerous lesions including adenomas in several studies (29,30). In a recent study conducted by Ahmed *et al* (31), a methylation panel including the *SEPT9* gene was analyzed in CRC patients, and concluded that *SEPT9* promoter methylation is a promising biomarker with the ability to discriminate CRC tissues as well as adenomas, from normal mucosa.

The *NTRK* neurotrophin receptor family includes *NTRK1* (*TrkA*), *NTRK2* (*TrkB*) and *NTRK3* (*TrkC*), which, in conjunction with their ligands (*NGF*, *BDNF* and *NT4/5*, *NT-3*, respectively) are important in development of the nervous system (32). It has been demonstrated previously that *NTRKs* have oncogenic effects in some cancer types, such as breast cancer and liver cancer (33). Recent studies have demonstrated that *NTRK1* and *NTRK3* may be dependent receptors, which depend on availability of their ligands to select their specific cell signals; these receptors are defined as having the ability to induce opposite effects in the presence or absence of their ligands (34,35). The availability of the ligand leads to the transduction of a positive cellular survival or differentiation signal, whereas the induction of apoptosis is a result of the absence of ligand (36). Observations which have demonstrated *NTRK3* is a beneficial prognostic factor in certain cancers, including melanoma and medulloblastomas, indirectly support that *NTRK3* has a dependence receptor role and is a conditional tumor suppressor gene (37,38). Therefore, these findings suggest that *NTRK3* may act as a conditional tumor suppressor gene in CRC.

Specific somatic missense mutations in *NTRK3*, which probably inhibit its function, have been identified in colorectal cancer, as well as breast, lung, and pancreatic cancers (39,40). Considering the possibility of *NTRK3* as a CRC tumor suppressor gene, based on the discovery of its mutant and methylated forms in CRC, Luo *et al* (41) conducted a study in order to define the effect of aberrant methylation on *NTRK3* expression, and also to define whether *NTRK3* has oncogenic or tumor suppressor functions in CRC cell lines. The authors concluded that aberrant methylation of *NTRK3* is prevalent in CRC and adenomas that consequently silences its expression, which suggests its tumor suppressor role. The results exhibited *NTRK3*'s function as a dependent receptor which means that binding its ligand, *NT-3*, it can induce proliferation while the absence of *NT-3* leads to *NTRK3*-mediated apoptosis. Overall, those findings suggested *NTRK3* as a novel conditional tumor suppressor gene in CRC.

The present study aimed to analyze the methylation status of *SEPT9* and *NTRK3* gene promoters in order to examine their ability to differentiate CRC tissues from normal mucosa, and to assess the validity of *NTRK3* as a methylation marker in clinical CRC samples.

Materials and methods

Study design. The present cross-sectional study was undertaken as a collaboration between the Immunology Research Center of Tabriz University of Medical Sciences, Imam Reza Hospital (Tabriz, Iran), Amiralmomenin Hospital (Tabriz, Iran) and Pasteur Institute of Tehran. Written informed consent was obtained from all patients participating in this study. The ethical protocol of the study was proved by the Ethical Committee of Tabriz University of Medical Sciences. A scheme of the study design is presented in Fig. 1.

Study population. Participants in this study were Iranians with the same ethnicity and geographical residency. Tumor and matched tumor-free margin samples were obtained from 45 colorectal cancer patients during surgery, which was a part of the routine treatment of patients. All patients were precisely identified as CRC patients on the basis of clinicopathological findings, all were candidates for cancer surgery, and underwent appropriate surgery at Imam Reza Hospital and Amiralmomenin Hospital, between 2014 and 2015. All the samples were referred to the laboratory under certain conditions with the complete patient information including clinicopathological and demographic data. The patients comprised of 19 males (42.2%) and 26 females (57.8%). None of the patients were undergone preoperative chemotherapy and/or radiotherapy and have no other malignancies. Tissue samples were separated into two distinct 45 tumor samples and 45 margin samples, which had been validated according to the pathological analysis.

Samples collection. Fresh tumor tissue and tumor-free margin tissue samples were collected during surgery in the Imam Reza Hospital, Tabriz and Amiralmomenin Hospital. Following resection, the tissue samples were immediately snap-frozen in liquid nitrogen and stored at -80˚C in the laboratory until further sample processing.

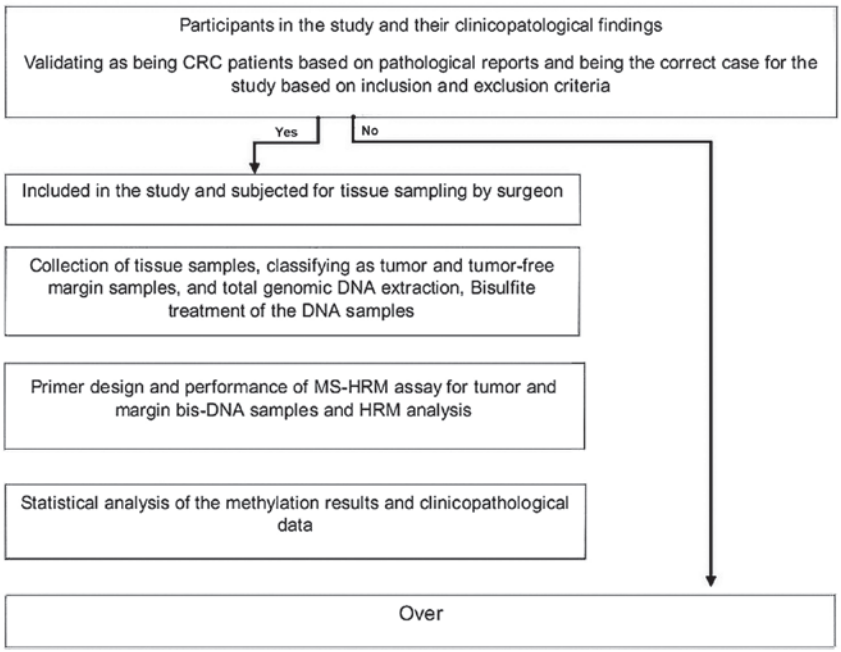

Figure 1. Schematic of the study design.

DNA extraction and sodium bisulfite modification. Total genomic DNA was extracted from tissue samples using *CinnaPure-DNA kit* (Cinna Colon, Iran) according to the manufacture's protocol. DNA concentrations were measured using a NanoDrop spectrophotometer, and then stored at -20˚C until the next step. Extracted DNA samples were excluded from the further analysis if the final concentration was <100 ng/μl, or the A260/A280 ratio was outside the range of 1.7-1.9. In the next step, total genomic DNA samples underwent sodium bisulfite conversion using a EZ DNA methylation-Gold kit (Zymo Research Corp. Irvine, CA, USA), according to the instructions provided by the company. The modified DNA samples were stored immediately at -20˚C.

Methylation specific-high resolution melting (MS-HRM). HRM primer pairs were designed for specific GC rich islands in promoter sequences of each gene according to the HRM primer design guidelines (Table I). Two specific locations were analyzed within the promoter region of *SEPT9* gene, and one location within the promoter region of *NTRK3* gene by MS-HRM assay. For each reaction, 2 μl of bis-DNA template was added to 10 μl of master mix (SYBR Premix Ex Taq™ II), and 2 μl of specific primer pairs with 6 μl double distilled water, then placed in Real Time PCR (Applied bio system, step one plus) with the following conditions: initial denaturation at 95˚C for 30 sec; and then 40 cycles at 95˚C of denaturation for 5 sec, appropriate annealing temperature for each primer set (Table I) for 30 sec, and extension at 72˚C for 30 sec. HRM analysis was done at a temperature range from 60˚C to 95˚C with the ramp rate of 0.3˚C/15 sec. The standard curves were included in each assay with DNA samples with known methylation ratios and then used to deduce the methylation ratio of each unknown sample. Using the HRM v.2.2 software (Applied Biosystems, Thermofisher Scientific, Waltham, MA, USA), melting curves were normalized relative to two normalization regions before and after the major decrease of fluorescence indicating the melting region of the PCR product. The output plots were in the shape of normalized melting curves. Considering the standard curves (0, 25, 50, 75, and 100%), HRM data for each unknown sample were classified into different ranges of methylation by three independent observers.

Statistical analysis. The analyzed data were found not to be normally distributed, therefore, nonparametric tests were used. Statistical analysis was performed in each group using the Mann-Whitney U and Kruskal-Wallis tests. The Spearman correlation coefficient test was used to analyze any correlation between clinicopathological findings of patients and gene specific methylation. Sensitivity and specificity of test were examined using ROC curve analysis. In all tests, $P<0.05$ was considered to indicate a statistically significant difference. SPSS version 22 was used for all statistical analyses (SPSS Inc., Chicago, IL, USA).

Results

Clinicopathological findings of patients. The mean age of the patients was 58.28 years (range 29-83 years), the median weight and height of patients were 71.11 kg (range 50-94 kg) and 165.51 cm (range, 150-196 cm), respectively. The tumor samples comprised of all CRC stages, however, the most prevalent stage was IIB. The pathological features of samples are displayed in Table II. The mean size of tumors was 5.5 cm (range 3-18 cm). Only 7 out of 45 patients were smokers.

Quantification of DNA methylation by MS-HRM assay. In order to determine the methylation level of *SEPT9* and *NTRK3* gene promoters, the MS-HRM assay, a semi-quantitative sensitive method was used. The assay optimized by using control dilution series including 0, 25, 50, 75, and 100% methylation

Table I. Primers used for MS-HRM assay.

Primer	Sequences (5'-3')	Ta˚C	Product size (bp)	Number of GC dinucleotide	Amplified region	
					Accession no.	Nucleotide numbers
SEPT9 (1st location)	F: CGGTGATAGAGAATTTTGTTTGGT R: CGACCTCAACCCCTCCC	60	178	11	NC_000017.11	77372911-77373089
SEPT9 (2nd location)	F: GACGTGTTGGAGAGGATTTTG R: CGAATACCCCTAACAAAATCCC	60	181	24	NC_000017.11	77373582-77373763
NTRK3	F: TGGTTCGGGAGATGTTTTT R: AAACGAACCAACAACTAATTAAA	58	164	16	NC_000015.10	88255602-88255765

Ta, appropriate annealing temperature. MS-HRM, methylation specific-high resolution melting.

controls. Representative results are shown in Fig. 2. For the first location of *SEPT9* gene, methylation was observed in 5/45 (11.11%) of normal adjacent samples and 25/45 (55.55%) of CRCs. In the second location of *SEPT9* gene, methylation was observed in 18/45 (40%) of normal adjacent samples and 42/45 (93.33%) of CRC samples. For *NTRK3* gene, the overall methylation level was high in both normal adjacent samples and CRC samples. The methylation status of the *NTRK3* gene promoter in the analyzed location was observed in 43/45 (95.5%) of normal samples and 45/45 (100%) CRC sampless, however, the mean methylation levels in tumor samples were much higher than those of normal adjacent ones.

The median methylation levels of *SEPT9* first location, *SEPT9 sec* location and NTRK3 in tumor samples was 24.16% (range 0 to 100%), 60.27% (range 0 to 100%), and 70.83% (range 25 to 100%) respectively, and in adjacent normal tissue was 3.61% (range 0 to 75%), 11.38% (range 0 to 75%), and 40% (range 0 to 75%) respectively. After doing statistical analysis it was shown that the methylation levels of *SEPT9* gene in both locations and *NTRK3* gene between tumor and matched normal adjacent tissue was significantly different (P<0.001; U Mann-Whitney test). Interestingly there was not any correlation between clinical and pathological features of patients with methylation levels of *SEPT9* gene in both locations and *NTRK3* gene, except for the first location of *SEPT9*, which was in correlation with primary tumor site. Methylation levels of *SEPT9* gene first location were different in distinct tumor sites. Moreover, there was no significant correlation between the two analyzed locations of *SEPT9* gene. Their methylation level was completely independent of each other.

In order to assess the applicability of *SEPT9* and *NTRK3* methylation as diagnostic biomarkers for CRC, the sensitivity and specificity of tests were analyzed using receiver operating characteristic (ROC) curve analysis. As shown in Table III and Fig. 3, acceptable sensitivity and specificity for *SEPT9* and *NTRK3* were resulted. When the cutoff of SEPT9 sec location methylation percentage was 31.25, the sensitivity, specificity, PPV, NPV, and accuracy were 84.40, 99, 90.36, 98.27, and 87.77% respectively. The results showed that the second location of *SEPT9* gene methylation could be better diagnostic marker for discriminating CRC tissues from matched normal adjacent with high accuracy. Likewise, high percentages for these parameters indicating diagnostic ability of *NTRK3* and *SEPT9* first location were also observed (Table III).

Discussion

The aberrant methylation in the promoter regions of genes is a prevalent event in a number of cancers including CRC (42). There are several methods to identify the methylation status of genomic DNA in specific sites; of those, MS-HRM is a highly sensitive semi-quantitative method, which can detect methylation level of specific region of bis-DNA precisely.

The MS-HRM assay was performed in order to identify the methylation status of *SEPT9* and *NTRK3* gene promoters. The methylation levels of *SEPT9* and *NTRK3* genes in CRC were high compared with matched normal tissue (P<0.001). There was not any significant association between clinical features and pathological findings of patients and hyper-methylation of analyzed genes in this study. The results of the

Table II. Clinicopathological findings of patients and their correlations with *SEPT9* and *NTRK3* methylation.

Clinicopathological features	Frequency	*SEPT9* methylation (first location) P-value	*SEPT9* methylation (second location) P-value	*NTRK3* methylation P-value
Age		0.31	0.17	0.18
<50	8			
>50	37			
Gender		0.76	0.73	0.60
Male	19			
Female	25			
Tumor location		0.04	0.62	0.38
Right colon	12			
Transverse colon	4			
Left colon	5			
Sigmoid colon	12			
Cecal	3			
Rectosigmoid	8			
Tumor size (cm)		0.51	0.34	0.06
<5	25			
>5	20			
Tumor grade		0.27	0.29	0.97
G1	20			
G2	23			
G3	2			
Tumor stage		0.61	0.81	0.11
Stage I	8			
Stage II	17			
Stage III	14			
Stage IV	6			
Smoking		0.73	0.63	0.87
No	38			
Yes	7			
Pre-operative hemoglobin (g/dl)		0.41	0.38	0.41
<12	26			
>12	19			

Table III. Diagnostic performance of *SEPT9* and *NTRK3* methylation.

Gene	AUC (95% CI)	Cutoff value (%)[a]	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
SEPT9 (first location)	0.732 (0.626-0.839)	6.25	55.6	99	85.53	95.29	72.22
SEPT9 (second location)	0.902 (0.835-0.970)	31.25	84.40	99	90.36	98.27	87.77
NTRK3	0.850 (0.771-0.929)	56.25	77.80	86.7	39.39	97.223	66.66

[a]Percentage of methylation. AUC, area under ROC curve; CI, confidence interval; PPV, positive predictive value; NPV, negative predictive value.

present study demonstrated that the first and second studied locations of *SEPT9* promoter region were not associated with age, gender, TNM stage, and grade of tumor, while the first location was just associated with tumor location. Similarly, *NTRK3* methylation was not associated with such clinical and pathological parameters. Previous studies have reported that

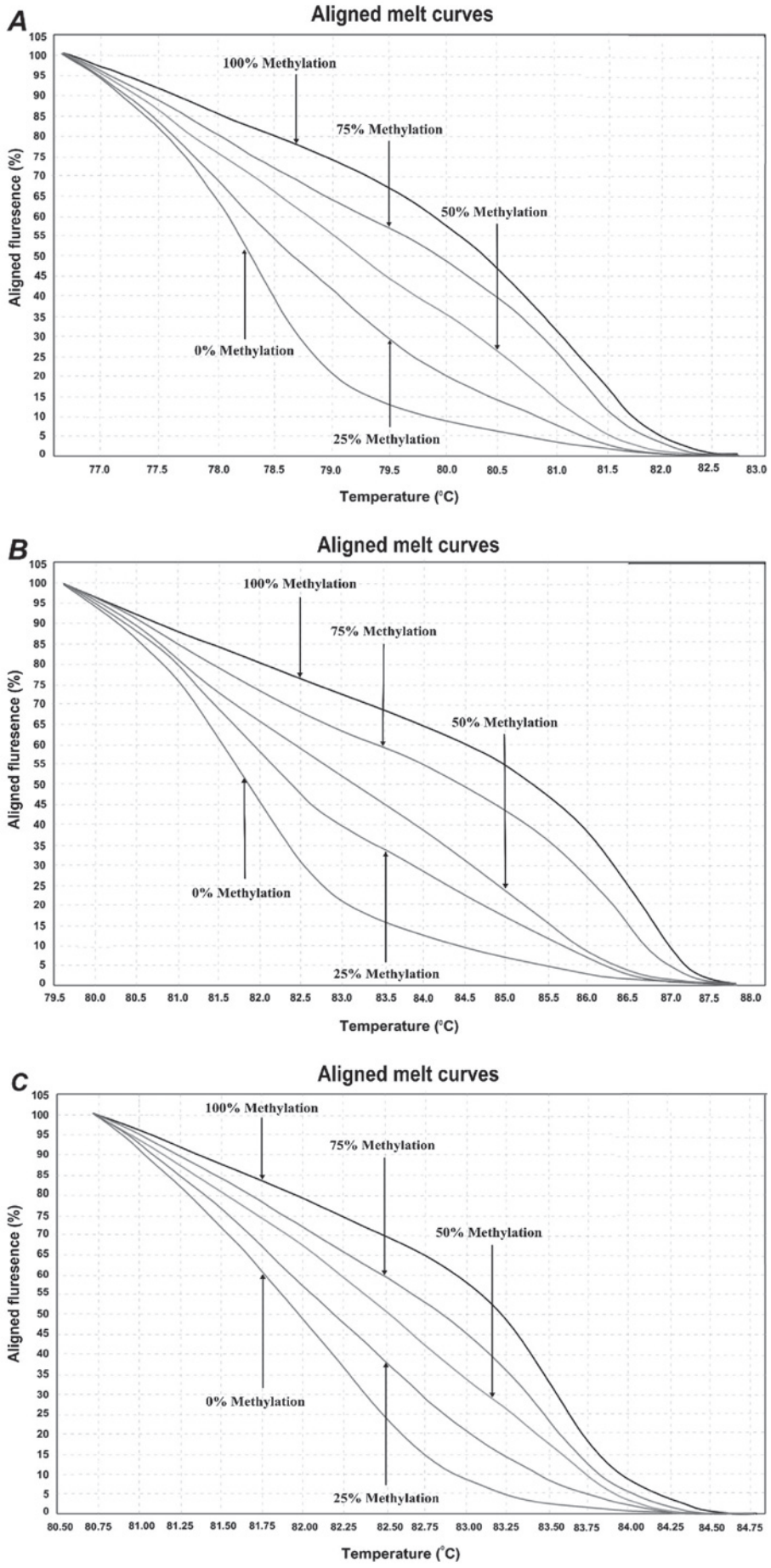

Figure 2. HRM aligned melt curves for three analyzed locations. (A) Control curves for SEPT9 gene 1st location. (B) Control curves for SEPT9 gene 2nd location. (C) Control curves for NTRK3 gene.

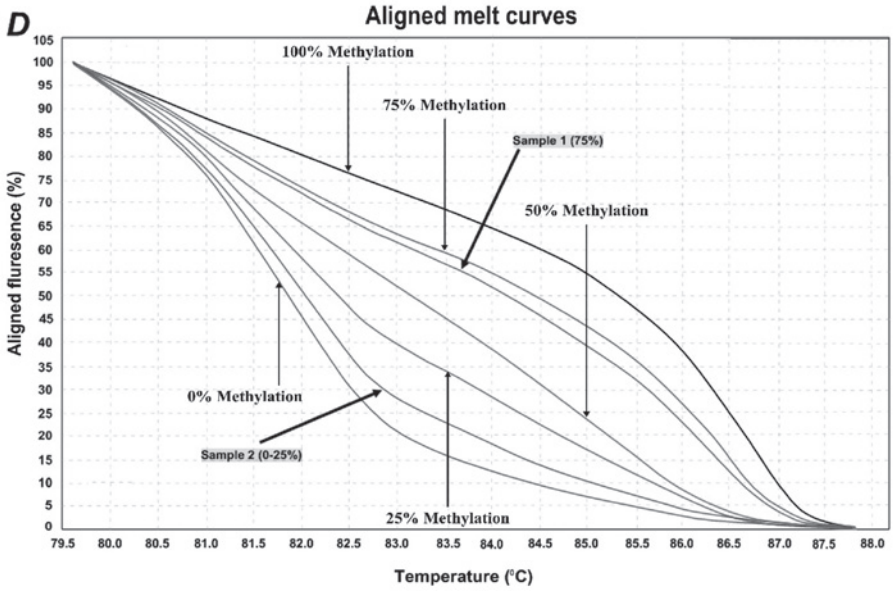

Figure 2. Continued. (D) Control curves and two sample curves for 2nd analyzed region of SEPT9 gene.

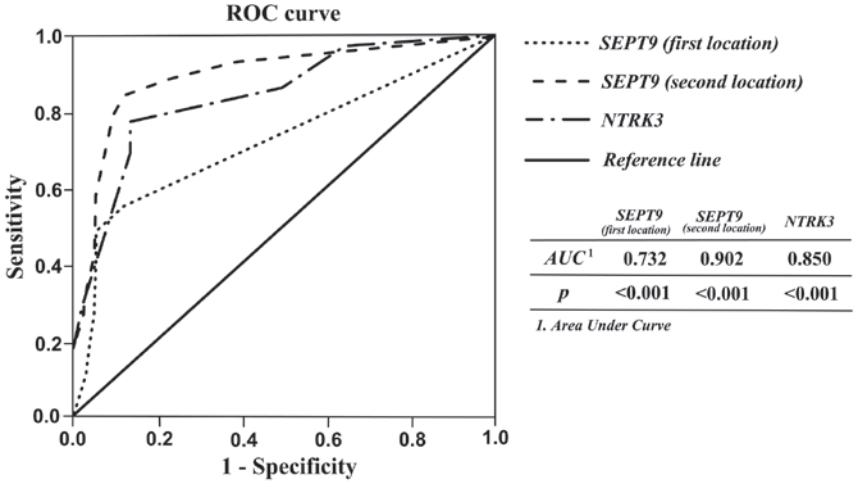

Figure 3. Receiver operating characteristic (ROC) curve analysis of SEPT9 and NTRK3 methylation using MS-HRM assay for discriminating CRC and matched adjacent normal samples.

the proportion of methylated *SEPT9* genes augmented with the progression of CRC (30,42), whereas our results demonstrate that this proportion of *SEPT9* was similar between different stages and tumor grades of CRC cases. These results are in agreement with the results of other similar studies such as Su *et al* (43). However, these findings may be biased due to the small number of cases included in the study. Statistical analysis indicated that there is no association between methylation level of first and second location of *SEPT9* gene, which suggests that their methylation process is independent of each other, however, the overall methylation in either tumor or normal matched adjacent tissue was higher in the second location in comparison to the first location, which may be due to the importance of this location in methylation-mediated silencing of *SEPT9*. In addition, the sensitivity and specificity of the second analyzed location for detection of CRC tissues form normal ones was higher than the first location. This finding suggests that this location may be more significant in *SEPT9* hypermethylation and the development of CRC, and can detect tumor tissues more precisely then first location. In an study established by Wassekort *et al* (44) it has been shown that hypermethylation in a specific CpG island of *SEPT9* promoter (including our studied locations) is probably an early event in adenocarcinoma progression. Furthermore, Wassekort *et al* (44) have proved that there is a direct link between this region and the region cross-examined by Epipro-Colon test that detects methylation of *SEPT9* in cell free DNA. Our results, in concordance with the published data, supports that *SEPT9* methylation in this CpG island can be a useful biomarker in CRC diagnosis. Tóth *et al* (20) analyzed *SEPT9* methylation in both tissue and plasma of healthy, adenoma and CRC cases quantitatively, and detected methylated *SEPT9* in all tissue samples at different levels regardless of the group. Methylated *SEPT9* levels in CRC and adenoma tissue samples were not significantly different; however, its levels in healthy tissue samples were much lower and considerably distinct from

either adenoma or CRC (20). Overall, our findings supported previous studies for *SEPT9* gene being a promising marker for detection of CRC.

The *NTRK3* gene has been recently demonstrated to become hypermethylated and silenced in CRC cell lines, which suggests its role as a tumor suppressor gene in colorectal cancer (41). The present study analyzed *NTRK3* promoter methylation in order to determine whether it can act as a biomarker in diagnosis of CRC or not. Our results showed that the overall mean of methylation level in either normal tumor free tissue or CRC tumor tissue samples was high in which there were just two samples with 0% methylation. Although our results are similar to those of Luo *et al* (41) and obviously *NTRK3* promoter methylation is able to discriminate the tumoral samples from normal tumor free samples with acceptable sensitivity and specificity, however, its high level methylation in tumor adjacent tissue suggests that it may start methylation process far before the appearance of any pathological features in cancerous cells. However, this hypothesis should indeed be further analyzed with samples in very initial stages of carcinogenesis.

In conclusion, based on our findings, *SEPT9* methylation can be used as a diagnostic marker independent of different clinicopathological features of CRC patients. In particular, the second location is a promising candidate, considering its high sensitivity and specificity and also the accuracy of the test. For *NTRK3* gene, we suggest further analysis with large sample size and specially samples in very initial stages of colorectal carcinogenesis in order to define its potential as a diagnostic biomarker in CRC.

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