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Aberrant *SEPT9* methylation in plasma cell-free DNA of CRC patients.

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Abstract

Colorectal cancer (CRC) is one of the most common cancers worldwide. CRC develops from precancerous polyps in the colon or rectum and is preventable by an early diagnosis and with the removal of precursor lesions. Numerous genetics and epigenetic alterations transform benign polyps to malignant tumors by affecting different pathways. Over the past decade, increasing evidence represent the utility of cell-free DNA as a 'liquid biopsy' to supplement non-invasive biopsies for genetic and epigenetic characterization and monitoring of solid cancers. One of the epigenetic biomarkers that has gained more attention in CRC is aberrant DNA methylation of *Septin 9* gene. In this study we try to evaluate the methylation of *Septin 9* gene status in the cfDNA of the plasma in colorectal cancer patients. Plasma cell-free DNA samples were extracted from 30 patients with background of tumors or polyps and 30 samples from healthy individuals. *Septin 9* methylation analysis was performed by using the bisulfite specific high resolution melting analysis. The result showed a sensitivity and specificity of 10% and 53.33%, respectively. In conclusion, our results demonstrated that *Septin 9* DNA methylation in plasma determined by THP and BSP-HRM had not have sufficient accuracy.

Keywords: Colorectal, Cancer, Methylation, *SEPT9*.

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Introduction

Colorectal cancer (CRC) is a malignancy that originates from the mucosal layer of the colon or rectum and adenomatous polyps [1]. CRC is the third and fourth most common cancer in men and women, respectively [2]. This causes 61,000 deaths annually around the world [3]. One of the most virulent tumors which has a second rank is CRC with an incidence of 13.1% in Europe and first ranked is belong to lung cancer [3]. A large number of patients are under the age of 50 years [4]. Screening of CRC is affordable cost which could be compared with other preventive method such as therapy of moderate hypertension [5]. CRC develops from precancerous polyps in the colon or rectum and is preventable and curable by an early diagnosis and with the removal of premalignant polyps. If tumor of CRC patients would be detected at early stage, the chance of survive will be increased. Adenomas as a premalignant lesion have a key role to diagnostic CRC [6]. Two approaches which are in used for CRC screening tests could be categorized: first on non-invasive tests for diagnosing primary cancer, such as guaiac fecal occult blood test (gFOBT), fecal immunochemical test (FIT) and stool DNA tests; second approach is invasive tests which could detect advanced lesions and cancer, such as double-contrast barium enema, colonoscopy, flexible sigmoidoscopy and virtual colonoscopy [7-9]. Colonoscopy tests cost a lot. Every 1,000 cases of colonoscopy, approximately one to five cases are associated with serious side effects [10]. In colonoscopy, tissue biopsy can be biopsied, which is a standard golden method for detecting colon cancer [11].

Today, various tissue analyses are done to diagnose and screen for CRC. One of these methods is genetic analysis [12]. Noninvasive biomarkers could be found in the stool or in plasma of patients and they are extremely sensitive and specific to evaluate genetic, epigenetic or protein markers [9,13-15]. Generally, the pathogenesis of CRC can be attributed to genetic and epigenetic changes in colon epithelium cells. Various mutations lead to genomic instability and play an important role in the development of CRC [16]. Chromosomal instability and micro-satellite molecular pathway involved in the development of CRC [17]. The methylation of genes as an epigenetic process could have a potential role in colorectal carcinogenesis [16-19]. One of the epigenetic biomarkers that has gained more attention in CRC is aberrant DNA methylation of *Septin 9* (*SEPT9*) gene.

Methylated *SEPT9* could be detected on cell-free tumor DNA. Almost every report on circulating DNA identifies apoptosis or necrosis or both as the main source of free circulating tumor DNA in serum and plasma [20-23]. Septin 9 proteins belong to upper class of P-loop GTPases and they are a group of GTP-binding proteins. Also, one of the main factors of cell division in yeast is *Septin* genes [24]. *SEPT9* has a major task in many cellular processes, like providing strength to the cell wall, recruiting proteins to specific subcellular locals to serve as scaffolds, making membrane diffusion barriers to create separate cellular domains and they have a role in cell polarity determination [24,25]. The molecular functionality of *Septin 9* (*SEPT9*) is not still discovered tumorigenesis of colon; the mentioned gene contains 18 unique transcripts which is encodes 15 polypeptides and generated by alternative splicing and its research has not been completed [26]. Methylated *SEPT9* (m*SEPT9*) has found in CRC cases and patients with precancerous lesions such as adenomas [21-25,27]. The objective of this research was to study aberrant DNA methylation of *SEPT9* gene in plasma of patients with pioneer lesions of CRC.

Materials and Methods

Study participants

This was a case-control study. Patients with sporadic CRC who participated in this study were recruited consecutively from April 2015 to March 2017. CRC tissues were collected during colonoscopy from 60 patients referred to Reza Radiotherapy and Oncology Center (RROC, Mashhad, Iran). In total, 30 polyp/tumor positive patients and 30 patients with normal colons diagnosed by colonoscopy were enrolled in this study. Histopathology reports were assessed to determine polyp/tumor characteristics. Patients with prior colorectal resection and history of any cancer or chemotherapy or radiation therapy were excluded from this study. In order to reduce bias, we designed this experiment as a blinded assay and samples were randomly coded before processing. All sample collection and preservation were taken care of by an individual who did not participate in the follow-up studies. All patients gave informed written consent to participate and to have their biologic specimens analysed. The study was approved by the Ethical Committee Mashhad University of Medical Sciences, Iran.

Collection of plasma

Five ml peripheral blood was collected from patients and healthy individuals into EDTA tubes and kept at room temperature (18-22°C). Plasma was separated by double centrifugation (800 g; 10 min, separation, 1600Xg; 10 min), no more than 2 h after blood draw. Plasma aliquots were immediately frozen at -70 °C.

Cell-free DNA extraction (cfDNA)

cfDNA purification was performed by the standard Triton/Heat/Phenol protocol (THP) method, which removes proteins from nucleic acids by mixture of phenol-chloroform-isoamyl alcohol. Briefly, in this method 500 µl of plasma was mixed with 5 µl Triton X-100 (Applichem, Germany) and heat denatured at 98°C for 5 min. Samples were placed on ice for 5 min, then extracted with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1, v:v:v), saturated with 50 Mm tris-Cl, pH 8.0 and centrifuged for 10 min at 14,000Xg. The aqueous phase was precipitated for 2 h with × 2.5 volume of 100% ethanol at -70°C. The DNA pellet was washed with 1 ml ethanol 70%, air-dried and re-suspended in 50 µl of AE buffer (10 mM tris-Cl, 0.5 mM EDTA; pH 9.0) and incubated overnight at 37°C.

Bisulfite treatment

Twenty µl extracted cfDNA undergone sodium bisulfite conversion and DNA recovery using the EpiTect Fast Bisulfite Conversion Kits (Qiagen, Germany) according to the manufacturer's instructions.

Methylation analysis

Methylation analysis was performed by bisulfite specific high resolution melting analysis (BS-HRM) consisting of PCR amplification of bisulfite-modified DNA. The primers used to amplify bisulfite-treated DNA were *SEPT9*-F 5'-TTTATTTAGTTGAGTTAGGGGGTTTA-3' and *SEPT9*-R 5' AACCCAACACCCACCTTC-3', designed to amplify both methylated and unmethylated bisulfite-treated DNA that did not amplify unmodified genomic DNA.

PCR amplification and HRM analysis were carried out sequentially on a light Cycler® 96 System (Roche, Germany). PCR was carried out in a 10 µl total volume using HiFiSYBR Green Master Mix (Farabin, Tehran), consisting of 2.5 µl of bisulfite modified template, 0.2 µg/µl BSA and 300 nM of each primer. The amplification run was 15 min at 95°C, followed by 45 cycles of 20 s 95°C, 15 s at the primer annealing temperature (60°C) and 15 s at 72°C. HRM analyses were performed at the temperature ramping from 65 to 97°C. Florescence acquisition setting was carried out at temperature recommended by the manufacturer. 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 the melting of the PCR product using the software version 1.1 provided with the LightCycler® 96 System.

Statistical analysis

The sensitivity and specificity (with 95% confidence interval (CI)) of the *Septin 9* hypermethylation of cfDNA plasma were calculated. To compare characteristics of the different groups of patients and samples, t-test for continue variables, Chi-square test and Fisher exact test were used for categorical variables. Statistical analyses were performed using SPSS version 13.0. All values were two-sided and P value<0.05 was considered to indicate a statistically significant difference.

Results

Patient and lesion characteristics

The clinical characteristics of the 60 patients included in this study were shown in Table 1. There was no significant difference with respect to gender and bone mass index (BMI) between cases and controls (p value>0.05). The BMI of individuals with polyps was 24.99%, which was not significantly different from the bodyweight of healthy individuals, which was 25%. In this research, patients with polyps had a weight loss of 20%. 16.66% of those were close to their ideal weight, 40% overweight, and 23.33% remained very obese.

SEPT9 methylation status

Figure 1 illustrates the comparison of the melting profiles of PCR products from samples with profiles specific for PCR products derived from methylated and unmethylated control DNAs.

Our results showed methylated *SEPT9* test had a sensitivity and specificity of 10% and 53.4% in patients' plasma with polyps/tumor, respectively. Statistical test analysis revealed that *SEPT9* methylation in plasma was not significantly different in patients with control groups (P>0.05) as shown in Table 2.

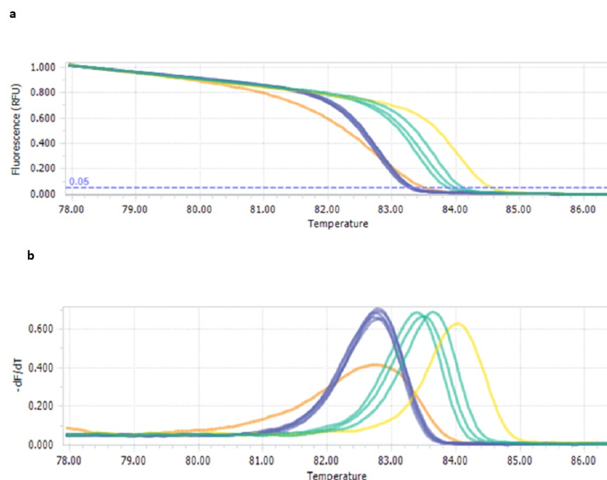

Figure 1. a) Normalized melting curve. b) Normalized melting peak. 100% Unmethylated DNA controls (orange), 100% methylated DNA control (yellow), Unmethylated sample (purple), Methylated sample (green).

Table 1. Patient and lesion characteristics.

Characteristics	Polyp/tumor	
	Negative	Positive
Sex		
Female	13 (43.33%)	12 (40%)
Male	17 (56.66%)	18 (60%)
Age group (y)	50.56 (15-79)	58.66 (30-76)
Body mass index (kg/m^2)		
Underweight (BMI<18.5)	0	6 (20%)
Healthy weight (BMI: 18.5-24.9)	9 (30%)	5 (16.66%)
Over weight (BMI: 25-29.9)	12 (40%)	12 (40%)
Obese (BMI of 30 or greater)	9 (30%)	7 (23.33%)
Hx. of drug intake		
Yes	1 (3.33%)	3 (10%)
No	29 (96.66%)	27 (90%)
Hx. of smoking		
Yes	8 (26.66%)	9 (30%)
No	30 (100%)	29 (96.66%)
Location		
Ascending colon	7 (18.91%)	4 (10.81%)
Rectum	-	7 (18.91%)
Sigmoid	-	17 (45.94%)
Transvers colon	-	1 (2.7%)
Descending colon	-	5 (13.51%)
Cecum	-	3 (8.1%)
Results of pathology	-	
Tubular adenoma	-	17 (56.66%)
Tubulovillous adenoma	-	5 (16.66%)
Vilous adenoma	-	0 (0%)
Hyperplastic polyp	-	3 (10%)
High grade adenoma	-	0 (0%)
Adenocarcinoma	-	5 (16.66%)
Adenoma size ≥ 1 cm	-	18 (60%)

Table 2. The performance of SEPT9 methylation test in plasma samples of CRC patients.

Characteristics/Polyp/Tumor	Positive (Methylated)	Negative (Un-methylated)	Sensitivity	Specificity	P-value

Positive	3	27	10%	53.33%	0.7
Negative	14	16			

Discussion

In this study, we aimed to assess the potential role of aberrant *SEPT9* promoter methylation changes in cfDNA released by tumor cells in different forms and at different levels in the blood circulation of CRC patients. We demonstrated that there was not significantly a higher frequency (P value>0.05) of *SEPT9* methylated DNA in plasma of patients with polyps/ tumor versus healthy individuals with a sensitivity and specificity of 10 and 53.4%, respectively.

According to several comprehensive screening researches, patients have early screening test, would have more chances for surviving compared to those who did not undergo any screening test [28-31]. A study performed in 2014, proved that most of the patients (83%) were willing to accept m*SEPT9* examine which is higher than colonoscopy (37%) and finally stool test (15%) [32]. According to studies conducted by Muller et al. a high degree of *SEPT9* sensitivity in plasma has made it a better way to detect CRC than FBOT and CEA [33]. Song et al. accomplished a *SEPT9* gene methylation test to diagnose CRC. A huge variety in sensitivity from 48.2% to 95.6% was detected as well as specificity from 100% to 80% [34]. In a study by Fu et al. sensitivity and specificity of 61.22% and 93.7%, for methylated *SEPT9* (m*SEPT9*) in plasma CRC cases, was reported respectively. They also displayed that plasma m*SEPT9* in monitoring CRC recurrences or metastases were reliable marker [35]. Besides, Wu el al. validated a simplified *SEPT9* gene methylation assay in 1031 subjects in Chinese patients. The sensitivity and specificity for CRC detection was 76.6% and 95.9%, respectively. Their results indicated a satisfactory detection rate for all stages of CRC, including early stages [36]. Toth et al. assessed *SEPT9* methylation in both tissue and plasma of healthy individuals, adenoma and CRC patients, and detected the methylated gene in all tissue samples at different levels regardless of the type. They realized that methylated *SEPT9* levels in CRC and adenoma tissue samples were not significantly different; however, its levels in adenoma or CRC cases were much higher and considerably distinct from healthy tissue samples [37]. Previously in a study by Lee et al. they showed that fecal immunochemical test also exhibited a high sensitivity for colon cancer similar to plasma *SEPT9* methylation [38]. The sensitivity of plasma m*SEPT9* for CRC was consistent with the data in a quantitative meta-analysis by Zhang et al., which showed that plasma methylated *SEPT9* had a sensitivity of 64% (95% CI: 59%-68%) for CRC detection in the Asian-based population [39]. Epi proColon® 2.0 CE is based on methylated *SEPT9* gene from the cfDNA in the plasma which is accessible in Europe and different nations such as china [40,41].

The low sensitivity and specificity in cfDNA-based studies could be due to different reasons. First, due to the very fragmented and low concentration of cfDNA in plasma, cfDNA extraction method plays a critical role. Some extraction methods such as THP generate a lower quality and quantity of cfDNA. Second, the specificity of *SEPT9* methylation in plasma is influenced by the background normal *SEPT9* methylation status. Aberrant cfDNA can be mixed by normal cell free DNA, shedded in the bloodstream (one percent of total cfDNA). cfDNA in the blood of cancer patients is not only representative of tumor derived DNA, but also of DNA released by healthy cells under different conditions [42,43]. Third, intertumoral heterogeneity could result in more complexity [44]. Therefore, these reasons may cause false positive/negative results.

This research demonstrates a sensitivity and specificity of 10 and 53.33%, respectively. Compare to other investigations, in the current study having several potential heteroduplexes generated by heterogeneous methylated CpG-rich amplicons is a challenge in BSP-HRM. It is hard to compare the homogenous methylated and unmethylated controls with melting HRM profile of heterogeneous methylated DNA samples [15]. Besides, the THP extraction method used in the current study could exacerbate the results and underestimate the sensitivity and specificity of *SEPT9* methylation in plasma.

In conclusion, our results demonstrated that *SEPT9* DNA methylation in plasma determined by THP and BSP-HRM had not have sufficient accuracy.

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