Genes & Genomics
https://doi.org/10.1007/s13258-017-0644-2

Online ISSN 2092-9293
Print ISSN 1976-9571

RESEARCH ARTICLE

BMP3 promoter hypermethylation in plasma-derived cell-free DNA in colorectal cancer patients

Parisa Rokni[1] · Afsaneh Mojtabanezhad Shariatpanahi[2] · Ebrahim Sakhinia[1,3] · Mohammad Amin Kerachian[2,4,5]

Received: 8 October 2017 / Accepted: 18 December 2017

Abstract

Detecting cfDNA in plasma or serum could serve as a 'liquid biopsy', for circulating tumor DNA with aberrant methylation patterns offer a possible method for early detection of several cancers which could avoid the need for tumor tissue biopsies. *Bone Morphogenetic Protein 3* (*BMP3*) was identified as a candidate tumor suppressor gene putatively down-regulated in colorectal cancer (CRC). In this study, we aimed to assess the potential role of *BMP3* promoter methylation changes in plasma DNA for detection of colorectal cancerous and precancerous lesions. Plasma DNA samples were extracted from 50 patients with histologically diagnosed polyps or tumor and 50 patients reported negative for polyps or tumors. The procedure consists of bisulfite conversion of the extracted DNA, purification of bis-DNA, and *BMP3* methylation status analysis by using the bisulfite specific high resolution melting analysis. This study demonstrated that there was a significantly higher frequency of *BMP3* methylated DNA in plasma in patients with polyps versus healthy controls with a sensitivity and specificity of 40 and 94%, respectively. In conclusion, our results demonstrated that *BMP3* DNA methylation in plasma had not have sufficient sensitivity and it should be used in combination with other biomarkers for the detection of CRC.

Keywords Biomarker · Colorectal · Cancer · Methylation

Introduction

Colorectal cancer (CRC), the major cause of morbidity and mortality, accounts for over 9% of all cancer incidences worldwide (Arnold et al. 2017; Haggar and Boushey 2009; Hessami Arani and Kerachian 2017; Lange and Laird 2013; Tanzer et al. 2010). Thus, early detection of CRC could have considerable clinical benefits including reducing mortality and morbidity (Lange et al. 2012). In this cancer, 5-year survival rates are 70 and 13% for regional and distant stages, respectively (Gonzalez-Pons and Cruz-Correa 2015). Due to the fact that CRC evolves primarily via the established adenoma-to-carcinoma pathway (Gonzalez-Pons and Cruz-Correa 2015), cancer screening could prevent cancerous formation and early treatment intervention for CRC patient (Levin et al. 2008). The American Cancer Society (ACS) has introduced CRC as a major priority since the application of current science and knowledge have such a great potential to prevent cancer, lower suffering and extend life expectancy (Levin et al. 2008). Although colonoscopy is the gold standard for CRC screening, this procedure is invasive, expensive, and patients suffer from inconvenience (Lange et al. 2012). Hence, there is a strong need for discovery of noninvasive detection method assays. In the recent years, several noninvasive tests have been developed for CRC screening (Ahlquist et al. 2012a). Noninvasive biomarkers are expected to be highly sensitive and specific to evaluate

✉ Ebrahim Sakhinia
esakhinia@yahoo.co.uk

✉ Mohammad Amin Kerachian
amin.kerachian@mail.mcgill.ca; kerachianma@mums.ac.ir

1 Connective Tissue Research Center, Department of Medical Genetics, Faculty of Medicine and Tabriz Genetic Analysis Centre (TGAC), Tabriz University of Medical Sciences, Tabriz, Iran

2 Cancer Genetics Research Unit, Reza Radiotherapy and Oncology Center, Mashhad, Iran

3 Division of Regenerative Medicine, Faculty of Medical and Human Sciences, School of Medicine, The University of Manchester, Manchester, UK

4 Medical Genetics Research Center, Mashhad University of Medical Sciences, Mashhad, Iran

5 Department of Medical Genetics, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran

Published online: 10 January 2018

genetic, epigenetic or protein markers that can be detected in the stool or in plasma of patients (Kim et al. 2008; Lange et al. 2012).

One of the main processes causing the initiation and progression of CRC is the accumulation of a variety of genetic and epigenetic changes in colon epithelial cells (Okugawa et al. 2015). Aberrant DNA methylation in promoter region of predominantly tumor suppressor genes occurs in the early stages of tumor development in precancerous lesions. This is a well-characterized event in tumor biology and is relevant to CRC development and progression (Grützmann et al. 2008; Mansour 2014; Tanzer et al. 2010).

Changes in methylation could be feasibly detected in both stool and blood-based samples, making these biological markers ideal candidates for a noninvasive test for early detection of CRC (Gonzalez-Pons and Cruz-Correa 2015).

Epi proColon® 2.0 CE is based on methylated *Septin9* (*SEPT9*) gene from the cfDNA in the plasma which is accessible in Europe and different nations such as china (Jin et al. 2015; Lamb and Dhillon 2017). Behrouz Sharif et al. represented that *SEPT9* promoter hypermethylation may serve as a promising biomarker for the detection of CRC development (Behrouz Sharif et al. 2016).

Circulating tumor DNA (ctDNA) in plasma or serum could serve as a 'liquid biopsy', for detecting abnormal methylation patterns. It offers a possible method for screening of several cancers and avoids the need for tumor tissue biopsies (Elshimali et al. 2013; Mansour 2014; Schwarzenbach et al. 2011; Zou et al. 2007). It has been shown cfDNA in cancer patients is a result of direct shedding from the primary tumor through apoptosis, necrosis or secretion, or potentially originates from free circulating tumor cells (Lange and Laird 2013; Schwarzenbach et al. 2011). DNA methylation patterns measured in peripheral blood not only have great potential to be informative biomarkers of cancer risk but also it is useful as a noninvasive test for CRC screening (Elshimali et al. 2013).

ColoGaurd™ kit is now a FDA approved DNA stool-based CRC screening test, combine aberrant *BMP3* and *NDRG4* promoter region methylation as well as *Kras* mutation and fecal immunochemical test (Ahlquist et al. 2012b).

Bone Morphogenetic Protein 3 belongs to the transforming growth factor-beta (TGFB) superfamily also known as osteogenin, which induces bone formation. It was identified as a candidate tumor suppressor gene putatively down-regulated in CRC (Loh et al. 2008). One of the first evidence for the importance of *BMP3* inactivation through methylation process in early polyp formation and colorectal tumor development has been published by Loh et al. (2008). Their observation suggested that *BMP3* is an attractive target for the future development of molecular blood and/or stool screening tests for the early detection of lesions with neoplastic potential.

The aim of this study was to investigate aberrant DNA hypermethylation of *BMP3* gene in plasma of patients with precursor 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 September 2015 to March 2017. CRC tissues were collected during colonoscopy from 100 patients referred to Reza Radiotherapy and Oncology Center (RROC, Mashhad, Iran). In total, 50 polyp/tumor positive patients and 50 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 analyzed. The study was approved by the Ethical Committee of Tabriz University of Medical Sciences, Iran.

Collection of plasma

5 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, 1600×*g*; 10 min), no more than 2 h after blood draw. Plasma aliquots were immediately frozen at − 70 °C because of cfDNA instability.

Cell free DNA extraction

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 14,000×*g*. The aqueous phase was precipitated for 2 h with X2.5 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

20 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). The BS-HRM protocol consists of PCR amplification of bisulfite-modified DNA (Wojdacz and Dobrovic 2007). The primers used to amplify bisulfite-treated DNA were *BMP3*-F, 5′-GGGTTAGYGTAG TAAGTGGGGTTGG-3′ and *BMP3*-R, 5′- AACCTACTC RCCCCAACCATAACTAAATACCC-3′, designed to amplify both methylated and unmethylated bisulfite-treated DNA that did not amplify unmodified genomic DNA. These primers located in CpG island region and consisted of 24 CpG sites. Genomic amplicon region of *BMP3* is shown in Fig. 1.

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 300 nM of each primer, 0.2 µg/µl BSA and 2.5 µl of bisulfite modified template. 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 *BMP3* hyper methylation 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 100 patients included in this study was shown in Table 1. There was no significant difference with respect to gender and bone mass index (BMI) between cases and controls (gender: $P=0.54$; BMI: $P=0.80$). Among the 50 polyps: 26% were located at proximal (ascending colon, hepatic flexure and transverse colon) and 74% were located at distal colon (descending colon, sigmoid, rectum, anal).

BMP3 methylation status

Amongst the 100 cfDNA only five samples were excluded in this study since they were not amplified properly by real time PCR.

Figure 2 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 *BMP3* test identified18 out of 45 patient plasma samples with a sensitivity of 40% and overall specificity of 94%. Statistical test analysis revealed that *BMP3* methylation in plasma was significantly different in patients with control groups (P < 0.05) as shown in Table 2.

Discussion

In this study, we aimed to assess the potential role of aberrant *BMP3* 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 significantly a higher frequency (P value < 0.05) of *BMP3* methylated DNA in plasma of patients with polyps/ tumor versus healthy individuals with a sensitivity and specificity of 40 and 94%, respectively.

Sensitivity is the main characteristic for screening tests because the major role of a screening test is to rule out diseases such as cancer or precancerous lesions. Although high sensitivity is the most important characteristic of a cancer-screening test, specificity is also important, since it affects

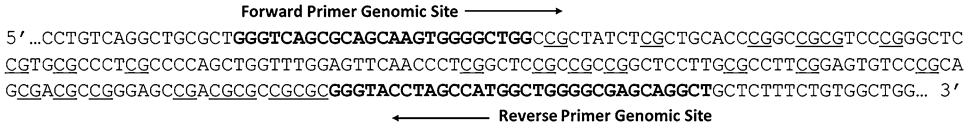

Fig. 1 BMP3 genomic amplicon. Forward and reverse primer sites (in bold), CpG sites (underlined)

Table 1 Patient and lesion characteristics

Characteristics	Polyp/tumor	
	Negative	Positive
Sex		
Female	25 (50%)	21 (42%)
Male	25 (50%)	29 (58%)
Age group (years)	50 (15–84)	59 (30–80)
Body mass index (kg/m^2)		
Underweight (BMI < 18.5)	0 (0%)	7 (14%)
Healthy weight (BMI: 18.5–24.9)	18 (36%)	12 (24%)
Over weight (BMI:25–29.9)	18 (36%)	16 (32%)
Obese (BMI of 30 or greater)	14 (28%)	15 (30%)
Hx. of drug intake		
Yes	1 (2%)	7 (14%)
No	49 (98%)	43 (86%)
Hx. of smoking		
Yes	9 (18%)	14 (28%)
No	41 (82%)	36 (72%)
Hx. of alcohol consumption		
Yes	1 (2%)	3 (6%)
No	49 (98%)	47(94%)
Location		
Anal	–	1 (1%)
Rectum	–	14 (21%)
Sigmoid	–	24 (37%)
Transvers colon	–	4 (6%)
Descending colon	–	9 (14%)
Ascending colon	–	7 (11%)
Cecum	–	6 (9%)
Results of pathology		
Tubular adenoma	–	27 (54%)
Tubulovillous adenoma	–	11 (22%)
Villous adenoma	–	1 (2%)
Hyperplastic polyp	–	3 (6%)
High grade adenoma	–	1 (2%)
Adenocarcinoma	–	7 (14%)
Adenoma size ≥ 1 cm	–	26 (52%)

the number of individuals who have positive test results (Berger et al. 2016).

Zou et al. (2007) evaluated *BMP3* gene methylated on 74 colorectal cancers, 62 adenomas, and 70 normal epithelia tissues. Methylation status was analyzed quantitatively and qualitatively and confirmed by bisulfite genomic sequencing. Methylation of *BMP3* was detected in 66 of cancers; 74% of adenomas; and 7% of normal epithelia ($P < 0.01$, cancer or adenoma versus normal). Loh et al. (2008) observed *BMP3* methylation in colorectal polyps and cancers, but not in normal mucosa samples, suggests that this may be an attractive target for the future development of molecular blood and/or stool screening tests for the early detection of lesions with neoplastic potential. Zou et al. (2012) used the QuARTS technology to quantify methylated *BMP3* gene on 91 DNA samples extracted from colorectal tissues, including 37 cancers, 25 adenomas, and 29 healthy epithelia. Comparing cancer/adenoma to healthy epithelia, AUC values were 0.89 for *BMP3* DNA methylation. Ashktorab et al. (2014) analyzed more than 21,500 CpG Islands methylation status using reduced representation bisulfite sequencing (RRBS) technique in colorectal cancer and adenoma tissues that were compared with DNA methylome from a healthy subject's colon tissue and peripheral blood DNA. They represented novel DNA methylation in six genes including *BMP3* that could be involved in CRC progression as it was significantly hypermethylated in tumor versus normal tissues. Houshmand et al. (2017) studied the methylation status of bone morphogenetic protein 3 (BMP3) gene, in tissue samples from patients with CRC obtained from colorectal surgery. They revealed that BMP3 methylation was detected with a sensitivity of 56.66% and a specificity of 93.3%, indicates its potentiality for early detection of CRC.

These tissue-based studies suggest that *BMP3* DNA methylation could be a potential biomarker for early detection of CRCs. Since released cfDNA in blood and DNA extracted from exfoliated gastrointestinal epithelial cells in stool reflects genomic alterations, using blood and stool samples could be beneficial sources to detect cancer (Galanopoulos et al. 2017; Park et al. 2017).

In Park et al. (2017) study, bisulfate-modified stool DNA obtained from 36 patients with advanced adenoma; 35 patients with CRC; and 40 endoscopically diagnosed healthy controls using CRC screening colonoscopy. Methylated *BMP3* were detected in 40.0% of CRC samples and in 33.3% of advanced adenoma samples with the specificity of 85%.

Ahlquist et al. reported sensitivity at 87% and specificity at 93% for methylated *BMP3/NDRG4/VIM/TFPI2* in stool DNA samples. In other study, Ahlquist et al. showed the sensitivity 85% and specificity 89% for *VIM/NDRG4/BMP3/TFPI2* genes for stool specimens Based on these studies and further investigations ColoGaurd™ kit was designed (Zhai et al. 2016).

The specificity degree of *BMP3* methylation (of colorectal cancerous cells) in stool or plasma is influenced by the *BMP3* methylation status of background normal DNA. The specificity and sensitivity of *BMP3* methylation (of cancerous cells) has been only studied in stool and tissue and there is no evidence for plasma. To address this issue, the authors studied the plasma BMP3 DNA methylation.

The relatively low sensitivity in this study could be due to different reasons. First, the very low concentration and fragmented cfDNA in plasma. Second, cfDNA with genetic and epigenetic alterations can be mixed by normal free DNA, released in the bloodstream (1.0% of total cfDNA) (Danese

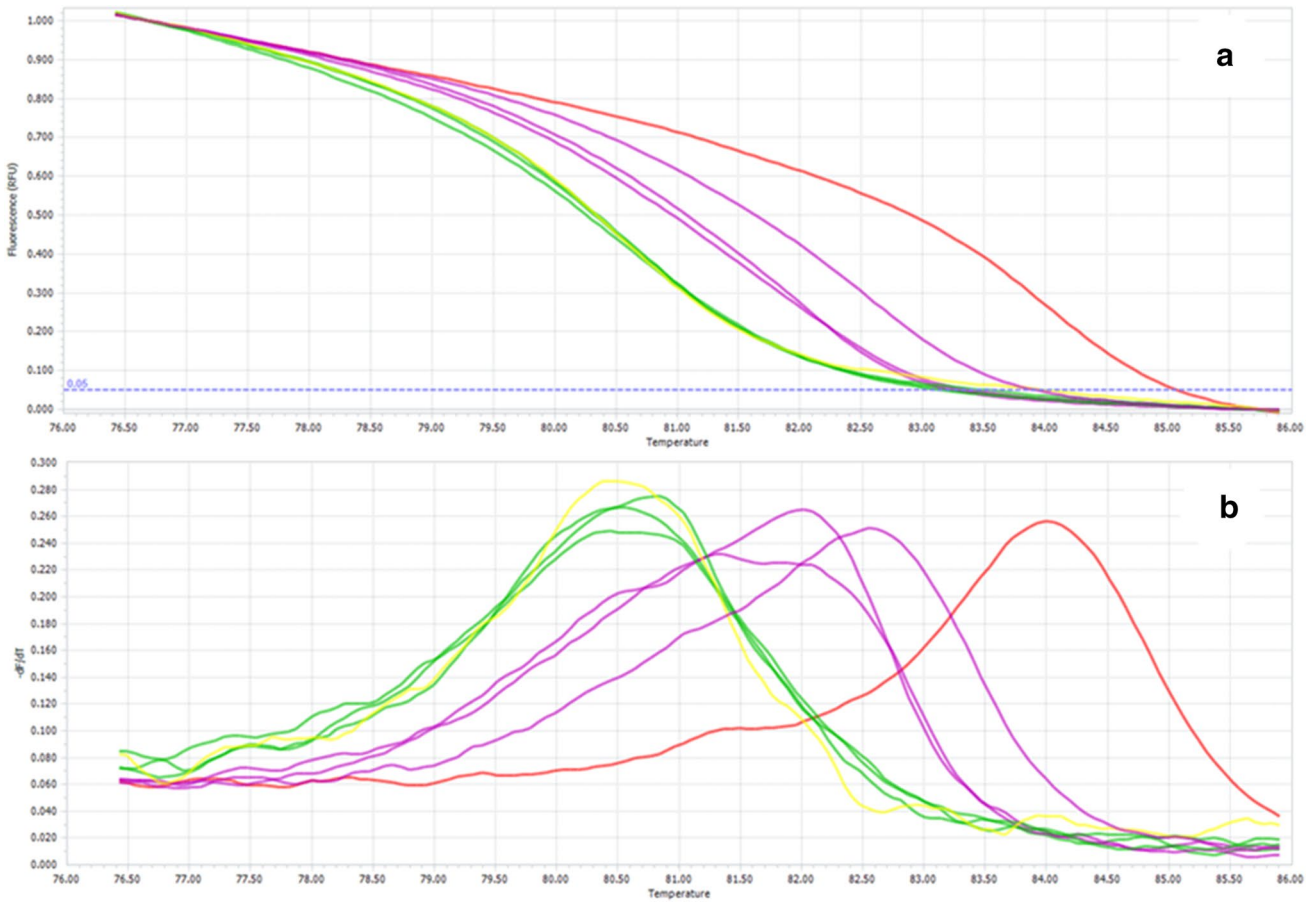

Fig. 2 **a** Normalized melting curve. **b** Normalized melting peak. 100% Unmethylated DNA controls (yellow), 100% methylated DNA control (red), Unmethylated sample (green), Methylated sample (purple). (Color figure online)

Table 2 The performance of BMP3 methylation test in plasma samples of CRC patients

Polyp/tumor	Characteristics					
	Positive (methylated)	Negative (unmethylated)	Unknown	Sensitivity	Specificity	P value
Positive	18	27	5	40%	94%	0.0002
Negative	3	47	0			

et al. 2015; Diaz and Bardelli 2014). Third, tumors, themselves are a mixture of different cancer cell clones (intertumoral heterogeneity) which could lead to more complexity (Kamat et al. 2006). Fourth, having a large number of potential heteroduplexes generated by heterogeneous methylated CpG-rich amplicons is a challenge in BSP-HRM. It is difficult to compare the multifaceted melting HRM profile of heterogeneous methylated DNA samples with homogenous methylated and unmethylated controls.

In conclusion, our results demonstrated that *BMP3* DNA methylation in plasma had not have sufficient sensitivity and it should be used in combination with other biomarkers for the detection of CRC.

Acknowledgements This study was supported financially by Tabriz University of Medical Sciences, and Reza Radiotherapy and Oncology Center, Mashhad, Iran. Our sincere thanks also go to Mr. Ebrahim Pouladin and Mrs. Nafiseh Shalchi for their close support in CRC research programs. We would also give special thanks to Dr. Abdorasoul Hayatbakhsh, Miss. Maryam Yassi and Adeleh Rezaie, Mrs. Neda Ziafati, and Zohreh Alizadeh for their cooperation in this study.

Funding This study was supported financially by Tabriz University of Medical Sciences (Grant # 54125388), and Reza Radiotherapy and Oncology Center, Mashhad, Iran.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consents were obtained from all individual participants included in this study.

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