Original Article

Methylation pattern of SFRP1 promoter in stool sample is a potential marker for early detection of colorectal cancer

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Abstract

Background: To setup a non-invasive genetic screening method for colorectal cancer, we evaluated the promoter methylation status of secreted frizzled-related protein1 (sfrp1) in stool samples of colorectal cancer with respect to a series of healthy individuals, using methylation-specific polymerase chain reaction.

Materials and Methods: In stool samples from 25 patients with colorectal cancer and 25 healthy control subjects, isolated DNA was treated with sodium bisulfite and analyzed by methylation-specific polymerase chain reaction with primers specific for methylated or unmethylated promoter sequences of the SFRP1 gene.

Result: Methylation of the SFRP1 promoter was present in the stool DNA of patients with colorectal cancer. A sensitivity of 52% and specificity of 92% were achieved in the detection of colorectal neoplasia. The difference in methylation status of the SFRP1 promoter between the patients with colorectal neoplasia and the control group was statistically highly significant ($P = 0.006$).

Conclusions: The results indicate that this DNA stool test of methylation of the SFRP1 promoter is a sensitive and specific method. It is assumed that the test is potentially useful for the early detection of colorectal cancer.

Key Words: Colorectal cancer, DNA methylation, secreted frizzled-related protein, stool DNA test

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INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer in the world, and is the second major cause of death from cancer in Europe and in the USA.[1,2]

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Detection of early disease and precancerous lesions Seems to be a key measure to reducing mortality rate from this disease.[3] CRC can be most effectively treated when diagnosed at an early stage.[4] Early detection can improve prognosis, but the recognition that virtually all CRCs arise from a discrete and accessible precursor lesion raises the prospect that cancer can essentially be prevented with an appropriate screening.[5] Yet, the acceptance of current screening methods is low. Only a minority (14 – 17 %) of average-risk adults older than aged 50 years undergo colonoscopy.[6,7] Fecal occult bleod testing (FOBT) is far more widely used;[8,9] however, tumors without bleeding can remain undetected.[10] The primary goal today is to identify

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the most sensitive and effective screening approaches that would maximize patient compliance. Colonocytes are continuously shed into the stool. Therefore, an analysis of their molecular and genetic alterations may aid CRC detection.[11] Studies aiming to make some correlation with various SNP have already been done in Isfahan.[12-14] However, it is a first attempt in using stool DNA methylation as a molecular marker in CRC detection in Isfahan. In a multicenter study in 2004, stool-based DNA tests were found to be 4 times more effective than FOBT for detecting CRC.[10] In addition, only one stool sample is needed for stool DNA tests compared with 3 samples for FOBT, and compliance and patient acceptance are clearly higher than for colonoscopy.[15,16]

The emergence of molecular stool testing provides a possible user-friendly alternative to conventional methods of CRC screening. One such strategy would be to develop tests for the detection of fecal DNA methylation patterns that will improve the sensitivity of non-invasive screening tests for colorectal neoplasia.[17,18] One of the principal epigenetic mechanisms known to be involved in carcinogenesis is the methylation of the cytosine residues of CpG-rich sequences (CpG islands) located within the promoter regions of genes regulating cell proliferation, apoptosis, and DNA repair. A number of genes have now been shown to be hypermethylated in CRC.[19] Silencing of SFRPs genes which are glycoproteins working as inhibitory modulators of a putative tumorigenic pathway (the Wnt signaling pathway) induced by promoter hypermethylation plays a key role in colorectal tumorigenesis.[20] In 1983, Feinberg and Vogelstein observed altered methylation of genes in colorectal tumors and adenomas.[21,22] In 2002, Suzuki *et al*. observed frequent promoter hypermethylation and silencing of SFRP genes in CRC and identified them as potential gatekeeper genes whose loss of function occurs early in CRC progression.[23] In present study, we studied the methylation status of SFRP1 gene in stool samples from patients with CRC and healthy individuals, using methylation-specific polymerase chain reaction (MSP), as a potential marker for stool-based early detection and early screening of colorectal cancer.

MATERIALS AND METHODS

Patients and collection of fecal DNA sample

DNA Samples: Human stool samples were collected from 50 individuals including 25 healthy volunteers and 25 patients with colorectal cancer before any treatment. About 5 g stool was collected from each individual. All the samples were collected in dry clean plastic containers. An informed consent was obtained from every subject prior to the study. Stools were collected prior to any preparation for colonoscopy or 4 - 5 days following this procedure. Tumor characteristics such as location, size, and stage, as well as, age, sex and other necessary information were recorded in a questionnaire. The stool specimens were stored at -20°C immediately after collection, to avoid potential enzymatic degradation of nucleic acids, and if longer storage was needed, then transferred to a -70°C.

DNA isolation from fecal samples

Samples were randomly coded before processing to ensure adequate blinding of the clinical information. DNA was isolated from stool samples (250 mg) by use of the QIAamp DNA Stool Mini Kit (Qiagene Germany) according to the manufacturer's protocol.

Bisulfite modification

DNA was chemically modified by sodium bisulfite to convert all unmethylated cytosines to uracils while leaving methyl cytosines unaltered (EpiTect Bisulfite Kit, Qiagen) and eluted in 50 µL of elution buffer.

Methylation specific PCR (MSP)

The bisulfate-modified DNA was used as a template for MSP as described previously.[24] Proper positive and negative controls were included in each batch of PCR reaction. Methylated and unmethylated primer sequences used in this study[25,26] as well as the annealing temperature and, product sizes are given in Table 1. For the MSP, 2 µL of bisulfite-converted DNA was used in each amplification reaction. PCR was performed in a reaction volume of 25 µL consisting of 17.875 µL ddH2O, 2.5 µL 10X PCR buffer, 0.2 mM dNTP mixture, 10 pM of each forward and reverse primers, and 2 units of TaKaRa Taq HS. Thermal cycling profile performed as follow: 95°C for 15 min, followed by 40 cycles at 94°C for 30 sec, specific annealing temperature (62°C for methylated primer pairs and 58°C for unmethylated primer pairs) for 30 sec, 72°C for 30 sec, and a final extension at 72°C for 5 min. The MSP products were then analyzed by 2.5% agarose gel electrophoresis.

Statistical analysis

Pearson chi-squared test was used to evaluate the association between the methylation status of the SFRP1 promoter in the DNA from all stool samples, as well as to evaluate the association between methylated SFRP1 promoter (positive or negative), tumor location (colon vs. rectum), patient group (control vs. CRC), and demographic variables, such as age and gender. $P > 0.05$ was considered to be significant. All statistical analyses were performed with the SPSS 13 software package (SPSS Inc., Chicago, IL).

Table 1: SFRP1 primers sequences, annealing temperature, and product sizefor MSP assays

Primer	Sequences (5-3)	Annealing temperature	Product size
SFRP1 MF	TGTAGTTTTCGGAGTTAGTGTCGCGC	62	126
SFRP1 MR	CCTACGATCGAAAACGACGCGAACG		
SFRP1 UF	GTTTTGTAGTTTTTGGAGTTAGTGTTGTGT	58	135
SFRP1 UR	CTCAACCTACAATCAAAAACAACACAAACA		

M - Methylated; U - Unmethylated; F - Forward; R - Reverse

Table 2: Characteristics of patients

Properties	Rates (%)
Tumor site	
Rectum	40
Cecum	25
Splenic flexture of colon	10
Ascending colon	15
Hepatic flexture	10
Tumor size	
3 cm	40
3 – 6 cm	25
> 6 cm	35
Gender	
Male	65
Female	35
Mean (sd) age	58 (12.87)

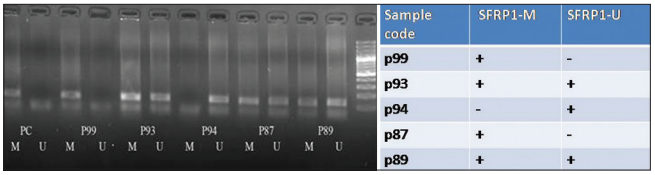

Figure 1: Detection of unmethylated (U) and methylated (M) SFRP1 in stool sample of CRC patients. P:patient, PC: positive control, M:methylated, U:Unmethyl

RESULT

Detection of methylated SFRP1 gene in fecal DNA

We assessed methylation status of SFRP1 promoter in fecal DNA from the patients and control groups by MSP reaction. The information of patients is shown in Table 2.

Patients had mean (sd) age of 58 (12.87) years. 35% of cases were female and 65% of patients were male. The most common tumor site was rectum (40%); other sites were cecum (25%), splenic flexture of colon (10%), ascending colon (15%), and hepatic flexture (10%), respectively. All the tumors were invasive adenocarcinoma. Tumor sizes were less than 3 cm in 40%, 3 - 6 cm in 25%, and more than 6 cm in 35%.

Methylation status of SFRP1 promoter for 13 patients was positive. In addition, for 23 subjects in control group, methylation of SFRP1 gene was negative. Based on our results, sensitivity of SFRP1 was 52% and specificity was 92%. Methylation status (positive vs. negative) of SFRP1 gene between CRC and control groups was significantly different (P value = 0.006).

DISCUSSION

The low acceptance of current screening methods has stimulated the search for a non-invasive, highly sensitive screening test. In analyzing various issues of CRC screening and the different screening tests, the following aspects need to be considered: (a), sensitivity and specificity, (b) safety, (c), acceptability, which often determines compliance, (d) cost, (e) efficacy (the extent to which medical interventions improve health under ideal circumstances), and (f) effectiveness, which is important because it indicates the accuracy of detecting and removing precancerous lesions.[27] Although currently colonoscopy is a gold standard procedure for the CRC diagnosis, but exhibits certain disadvantages like high costs, increased risk of perforation and bleeding, difficult preparation for the patients, and the need for sedation.[28] Disadvantages of FOBT are low sensitivity, low specificity, poor compliance, and the need for colonoscopy to confirm a positive test result.[29]

Stool-based DNA hypermethylation testing is a new, non-invasive method of colorectal cancer screening. It is easier to perform and is more sensitive than fecal occult blood testing, only a single stool sample is needed, does not require diet or medication restrictions, and evaluates the whole colon and rectum.[30] Aberrant Wnt signaling pathway is an early molecular event in 90% of CRCs, contributing to the growth, proliferation, and loss of apoptosis of tumor cells.[31] SFRPs are tumor suppressor proteins that contain a domain similar to one of WNT-receptor frizzled proteins (Fz) and may block Wnt signaling either by interacting with Wnt proteins to prevent them from binding to Fz proteins or by forming non-functional complexes with Fz.[32] Epigenetic inactivation of SFRP genes induced by promoter hypermethylation has been shown to play an important role in development of CRC by allowing constitutive WNT signaling.[33,34]

Detection of tumor-derived DNA alterations in fecal samples is an intriguing new approach with high potential for the non-invasive detection of CRC.[35] Methylation analysis of a number of gene promoters in DNA from fecal samples has been less comprehensively investigated, but has been suggested to be a sensitive diagnostic tool for colorectal tumor.[36] Our test indicated a sensitivity of 52% and specificity of 92% ($P < 0.006$). The presence of both methylated and unmethylated promoter sequences is in agreement with the heterogeneous mixture of dysplastic, tumor, and normal cells characteristically observed in early stages of carcinogenesis. Recently, Muller and colleagues[35] reported the detection of SFRP1 promoter methylation in the stool DNA of patients with CRC. In a preliminary setup with 10 CRC patients and 13 healthy control subjects, they achieved 90% sensitivity and 77% specificity, and in the fecal DNA from an independent test set of 13 patients with CRC and 13 healthy control subjects, a sensitivity of 77% and a specificity of 77% were obtained. In various stool-based studies using SFRP1 gene, different levels of sensitivity and specificity were obtained, mostly with satisfactory achievements.[36,37]

CONCLUSION

Hypermethylation of SFRP1 gene promoter in feces are novel epigenetic biomarkers of CRC and carried high potential for the remote detection of CRC as an non-invasive screening method.

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