ORIGINAL ARTICLE

DNA methylation analysis of secreted frizzled-related protein 2 gene for the early detection of colorectal cancer in fecal DNA

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ABSTRACT

Background: The early detection of colorectal cancer (CRC) with high sensitivity screening is essential for the reduction of cancer-specific mortality. Abnormally methylated genes that are responsible for the pathogenesis of cancers can be used as biomarkers for the detection of CRC. The methylation status of the secreted frizzled-related protein 2 (SFRP2) gene was evaluated for their use as a marker in the noninvasive detection of CRC. **Materials and Methods:** Methylation-specific polymerase chain reaction was performed to analyze the promoter CpG methylation of SFRP2 in the fecal DNA of 25 patients with CRC and 25 individuals exhibiting normal colonoscopy results. **Results:** Promoter methylation levels of SFRP2 in CRC patients and in healthy controls were 60% and 8%, respectively. Methylation of the SFRP2 promoter in fecal DNA is associated with the presence of colorectal tumors. **Conclusion:** Hence, the detection of aberrantly methylated DNA in fecal samples may present a promising, noninvasive screening method for CRC.

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

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INTRODUCTION

The incidence of colorectal cancer (CRC) and its mortality rate has increased in the recent years, and it gives rise to difficulties for many health systems in the world.[1] CRC affects close to 150,000 patients in the United States annually and it is the cause of nearly 50,000 deaths.[2] CRC patients have more chance to be treated if it is detected in the early stage of the disease,[3] therefore early detection can reduce mortality and improve survival rates.[4] Although there are many techniques to detect CRC such as the fecal occult blood test (FOBT) and colonoscopy that are more commonly used,[5] they have their limitation to detect, and majority of tumors can remain undetected and it can lead to overtreatment or undertreatment of disease with increasing false-positive and false-negative test results.[6,7] In addition, FOBT screening decreases only CRC mortality but not the incidence of CRC, and it is not able to detect precursor lesions.[7,8] Colonoscopy is an invasive method that causes adverse effects including postpolypectomy bleeding and perforation;[8] for this reason, the primary goal is to identify useful screening tools such that it could increase the sensitivity and specificity of screening without invasive actions.[8,9] Molecular and genetic study can play a key role in the detection of CRC because genetic alterations are the main causes of colorectal neoplasia.[10] Some studies have already tried to show the correlation between various SNP and CRC.[11-13] One thing is obvious enough that stool-based tests are a noninvasive method, but, in contrast, structural exams are invasive.[8] Scientists are hoping for improving CRC screening by potential benefits of stool-based DNA tests.[14] Interestingly enough, the number of genes silenced by epigenetic mechanisms is more than the number of genetic mutations in CRC that it put forward an important

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role of epigenetic alterations.[15] There are a great number of genes that will be used as DNA methylation biomarkers ahead that would increase the sensitivity of noninvasive screening tests for CRC.[9,16] SFRPs genes have a key role in the inhibitory modulators of a tumorigenic pathway (the Wnt signaling pathway), and hence silencing of the SFRP genes is the leading cause of Wnt pathway activation that gives rise to the genesis of the colorectal tumor.[17] It is said that loss of APC gene activity is a prevalent event in sporadic colorectal tumorigenesis that occurs in nearly 80% of cases and because it functions within the Wnt/β-catenin arm of the Wnt signaling pathway, hence it is likely to be expected that changes in this pathway such as silencing of SFRPs genes may be seen in colorectal tumor. In some papers, the secreted frizzled-related protein 2 (SFRP2) gene methylation has been demonstrated as the most sensitive single DNA-based marker in stool for identification of CRC due to the fact that the epigenetic inactivation of SFRP2 gene gives rise to the constitutive Wnt signaling in these putative precursors of CRC.[18-20]

The aim of our research is to study the methylation status of SFRP2 gene in stool samples from patients with CRC and normal cases, making use of methylation-specific polymerase chain reaction (MSP), as an effective way to screen and detect in the early stages of CRC.

MATERIALS AND METHODS

Fifty stool samples were collected from 25 CRC patients and 25 healthy volunteers, as control group without any history of familial cancer, according to their own colonoscopy. The experimental design was approved by the Ethics Committee of Isfahan University of Medical Sciences, and informed consent was obtained from each patient and healthy individual. All stool samples were stored in − 80°C after labeling them to prevent any enzymatic degradation of DNA.

DNA extraction was done by the use of QIAamp DNA Stool Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. The quality of the DNA extract was examined by spectrophotometry and gel electrophoresis. Then, all the DNA extracted were transferred to −20°C.

DNA treatment by EpiTect Bisulfit was used to convert all unmethylated cytosines to uracil while leaving methyl cytosines unaltered (EpiTect Bisulfite Kit, Qiagen) and eluted in 50 μL of elution buffer. We used myogenic differentiation gene as positive control due to the fact that this gene does not have any CpG island so that any cytosine will be converted to uracil after treatment by bisulfite. In addition, methylated DNA without any treatment by bisulfite was used as a negative control.

MSP was performed with specific primers for either methylated or unmethylated DNA, as previously described. Table 1 shows the MSP primers. Briefly, a 2 μg DNA sample was used in each amplification reaction. In addition, 17.87 μl O2HDD, 2.5 μl polymerase chain reaction (PCR) buffer 10X, 2-μl dNTP, 0.25 μl forward and 0.25 μl reverse primer, and 0.125 μl TakaRa Taq HS were used in MSP reaction. The MSP procedures for SFRP2 gene were performed as follows: 95°C 10 min, 95°C 45 s 45 cycles, 50°C 30 s 45 cycles (annealing temperature for unmethylated primer pairs), 62°C 30 s 45 cycles (annealing temperature for methylated primer pairs), 72°C for 30 s, and 72°C 5 min for final extension. We used negative and positive controls as described above.

Statistical analysis

Statistical analyses were carried out by the aid of a digital computer, using the Statistical Package for Social Science (version 13.0; SPSS Inc., Chicago, IL, USA) program. Pearson's Chi-squared test was used to assess the association between the methylation status of the SFRP2 promoter in the DNA from all stool samples, as well as to assess the association between methylated SFRP2 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.

RESULTS

In this study, the status of the SFRP2 gene methylation was assessed by MSP reaction in patients and control groups [Figure 1].

Table 1 summarizes the characteristics of the twenty patients in the study (65% males, 35% females); the mean ± standard deviation age was 58 years.

The methylation levels of the SFRP2 gene were assessed in the patient and control groups that give rise to the following findings: 12 from 20 patients were methylated and in

Table 1: Secreted frizzled-related protein gene 1 primers sequences, annealing temperature, and product size for methylation-specific polymerase chain reaction assays

Primer	Sequence 5′ to 3′	Annealing temperature	Product size
SFRP2 MF	GGGTCGGAGTTTTTCGGAGTTGCGC	62	138
SFRP2 MR	CCGCTCTCTTCGCTAAATACGACTCG		
SFRP2 UF	TTTTGGGTTGGAGTTTTTTGGAGTTGTGT	50	145
SFRP2 UR	AACCCACTCTCTTCACTAAATACAACTCA		

M – Methylated; U – Unmethylated; F – Forward; R – Reverse; SFRP – Secreted frizzled-related protein gene

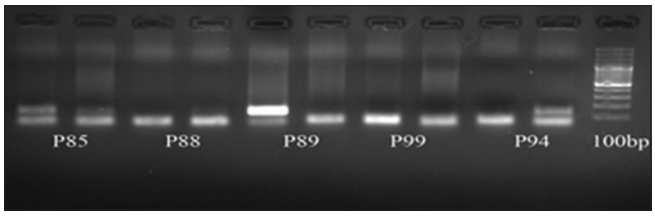

Figure 1: Methylation status of secreted frizzled-related protein 2 gene in stool samples

control group, unlike the patients, 2 from 20 individuals were methylated. For SFRP2 gene, sensitivity and specificity were 60% and 92%, respectively. Methylation status (positive vs. negative) of SFRP2 gene between CRC and control groups was significantly different ($P = 0.006$).

DISCUSSION

It is reasonable to screen CRC in individuals aged >50 years for reduction in the incidence and mortality of CRC. There is no doubt that genetic diagnosis may provide a noninvasive highly sensitive screening test. To find a proper screening test, it is necessary to consider some aspects of tests such as (a) sensitivity and specificity, (b) safety, and (c) acceptability, which often determine compliance, (d) cost, (e) efficacy (the extent to which medical interventions improve health under ideal circumstances), and (f) effectiveness, which are important because they indicate the accuracy of detecting and removing precancerous lesions.[21] There are several limitations for routine screening methods including colonoscopy and FOBT. Colonoscopy is an invasive method that patients may be not willing to do it and although FOBT is a noninvasive method, the sensitivity of FOBT is approximately 15–35% that could not detect those tumors without bleeding. Although colonoscopy is more sensitive, it is an invasive method that may cause perforation and bleeding and there are more disadvantages such as high costs, difficulty in preparation for the patients, and the need for sedation.[22] Epigenetic diagnosis is noninvasive and highly sensitive in contrast to colonoscopy and FOBT screening. Highly sensitive, specific, and easily analyzable markers are required for noninvasive stool-based CRC screening. Several studies have been done in detecting DNA mutations in the feces of CRC patients. Lu *et al*.[23] analyzed stool samples from 56 patients. This study assayed methylation status of SFRP2, GATA4/5, NDRG4, and VIM genes. Sensitivity and specificity were 96.4% and 65%, respectively. There are limitations to use multitarget panels due to the high cost and a difficult collection process. It is estimated by a meta-analysis that there is an overall sensitivity of 62% and a specificity of 80% for colorectal neoplasia by using methylated genes in the feces of CRC patients. Wnt signaling pathway acts as an oncogenic way that aberrant activation of the Wnt pathway may give rise to the variety of human cancers, especially in CRC. SFRP2 gene, an important member of the SFRP family, functions as a negative regulator of the oncogenic Wnt pathway through competing with frizzled membrane-bound receptors that can use it as an appropriate marker in CRCs screening.[9] Müller *et al*.[24] reported that SFRP2 hypermethylation exhibits a sensitivity of 77–90% with regard to identifying patients with CRC. Huang *et al*.[25] reported that methylation of SFRP2 occurs in 94.2% of the patients with CRC, with occurrences of 52.4, 37.5, and 16.7% in adenomas, hyperplastic polyps, and ulcerative colitis, respectively. In the recent study, it has been demonstrated the status of methylation of SFRP2 gene in stool DNA of the patients with CRC that could be used as a noninvasive and high sensitive method to choose for screening test in future.[26] Here, we demonstrate that SFRP2 is significantly hypermethylated and downregulated in CRCs when compared with nontumor samples. For SFRP2 gene, sensitivity and specificity were 60% and 92%, respectively, and methylation status (positive vs. negative) of SFRP2 gene between CRC and control groups was significantly different ($P = 0.006$). In summary, the detection of tumor-derived DNA alterations in the stool is a fascinating new approach with a considerable potential for the noninvasive detection of CRC. Our results demonstrate that the hypermethylation of SFRP2 in fecal samples shows promise for the accurate detection of CRC.

CONCLUSION

Incidence of CRC is increasing and effective early detection of colon cancer would be beneficial to reduce mortality and costs. DNA methylation, one of the molecular mechanisms in carcinogenesis of colon cancer can be used as biomarker to achieve our aim to early diagnosis of CRC. DNA methylation markers paved the way for developing noninvasive diagnostic assays. The methylation status of SFRP2 gene has a high potential to use as non-invasive method to detect CRC patients in the early stages of tumor. We conclude that the methylation pattern of SFRP2 gene in stool DNA may offer a good alternative in the early noninvasive detection of CRC.

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Conflicts of interest
There are no conflicts of interest.

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