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Research Paper

Combination analysis of hypermethylated SFRP1 and SFRP2 gene in fecal as a novel epigenetic biomarker panel for colorectal cancer screening ☆

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Abstract

Objective: To investigate the feasibility of the combination of detecting hypermethylated secreted frizzled-related protein 1(SFRP1) and secreted frizzled-related protein 2(SFRP2) in feces as a panel of biomarkers for colorectal cancer(CRC) screening. **Methods:** Methylation-specific PCR(MSP) was performed to analyze methylation status of SFRP1 and SFRP2 in a blinded fashion in tumor tissues and in matched stool samples from 39 patients with primary CRC, 34 patients with adenomas, 17 patients with hyperplastic polyps and 20 endoscopically normal subjects as normal controls. Simultaneously we analyzed the correlation of hypermethylated SFRP1 and SFRP2 with the clinicopathological features of CRC. **Results:**Hypermethylated SFRP1 was detected in 92.3%, 76.5%, 47.1% of tissue samples and in 89.7%, 64.7%, 35.3% of matched fecal samples from CRC, adenoma and hyperplastic polyp, respectively. Hypermethylated SFRP2 was detected in 87.2%, 67.6%, 35.3% of tissue samples and in 82.1%, 55.9%, 29.4% of matched fecal samples from CRC, adenoma and hyperplastic polyp, respectively. Of these two genes, at least one hypermethylated was 94.9%, 82.4%, 52.9% in tissue samples and 92.3%,73.5%, 47.1% in matched fecal samples from CRC, adenoma and hyperplastic polyp, respectively. In contrast, no hypermethylated SFRP1 and SFRP2 were detected in mucosa tissues of normal controls, only 2 cases of fecal samples was detected with hypermethylated SFRP1 and another 1 case was detected with hypermethylated SFRP2. Moreover, no significant associations were observed between hypermethylated SFRP1,SFRP2 and clinicopathological features of CRC. **Conclusion:** Hypermethylation of SFRP1 and SFRP2 in feces are novel epigenetic biomarkers of CRC and carried high potential for the remote detection of CRC as non-invasive screening method, and combined analysis of hypermethylated SFRP1 and SFRP2 in fecal could further increase the detection rate of CRC and premalignant lesions.

Key words: Secreted frizzled-related protein 1(SFRP1); secreted frizzled-related protein 2(SFRP2); methylation; colorectal cancer; feces

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]. There were approximately one million CRC cases estimated world wide in 2006, resulting in more than 500,000 deaths[3]. However, its mortality rate has been decreasing over the last decade, and this may be attributable to more effective screening and surveillance[4]. CRC can be most effectively treated when diagnosed at an early stage[5]. 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 appropriate screening[6]. The primary goal today is to identify the most sensitive and effective screening approaches that will maximize patient compliance. 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

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fecal DNA methylation patterns that will improve the sensitivity of noninvasive screening tests for colorectal neoplasia, and moreover, will decrease both mortality and the incremental costs of treating CRCs. Methylation analysis of a number of gene promoters in DNA from stool samples has been less comprehensively investigated, but has been suggested to be a sensitive diagnostic tool for colorectal tumor[7-12]. Silence of SFRPs genes which are secreted 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[13]. Detection of hypermethylation of SFRPs genes in human DNA isolated from stools might provide a novel strategy for the detection and investigation of sporadic CRC. The combination analysis of aberrant methylation of SFRP1 and SFRP2 gene(two of SFRPs gene family member) we described here which are frequently hypermethylated in CRC, adenoma and Aberrant crypt foci(ACF), and thus the combination assay of hypermethylated SFRP1 and SFRP2 gene is a potential epigenetic biomarker panel for colorectal cancer screening in fecal DNA.

MATERIALS AND METHODS

Patients and Collection of Tissue and Fecal DNA Samples

Colorectal mucosa specimens were routinely collected from 39 patients with sporadic CRC, 34 patients with adenomas(19 adenomas with size $\geqslant$ 1 cm; high-grade dysplasia, 15 adenomas with size $<$ 1 cm; low-grade dysplasia), 17 patients with hyperplastic polyps and 20 endoscopically normal subjects undergoing endoscopy at the first affiliated hospital of Yangzhou university from March 2005 to February 2007. During the study the matched fecal specimens were collected and kept at 4℃ until being processed. Within 12 hours of collection, the specimens were washed with 1 × PBS and centrifuged at 1,800 rpm for 15 minutes to pellet the solid stool. The pellet of stool and tissue specimens were stored at –80℃. None of the patients had received chemotherapy or radiation therapy prior to surgery. All patients gave informed consent for their participation in the study and had been approved by the Ethical Committee of our university.

DNA isolation from tissue and fecal samples

Samples were randomly coded before processing to ensure adequate blinding of the clinical information. DNA was isolated from colonic tissues(5-10 mg) by use of the QIAamp DNA Mini Kit(Qiagen) and from stool samples(250 mg) by use of the QIAamp DNA Stool Mini Kit according to the manufacturer's protocol. H&E-stained sections from tissue samples were examined by an experienced pathologist to confirm the histological diagnosis.

Bisulfite modification

The DNA was chemically modified by sodium bisulphate to convert all unmethylated cytosines to uracils while leaving methylcytosines unaltered (EpiTect^{R} Bisulfite Kit, Qiagen), and eluted in 50 μL of elution buffer.

Methylation specific PCR(MSP)

The bisulfite-modified DNA was used as a template for MSP as described previously[13]. SFRP1 and SFRP2 gene were examined, and template-free distilled water was included as a negative control for amplification. The sequences of primers were also as reported previously[14,15](***Tab 1***).

Tab 1 **SFRP1 and SFRP2 gene primers sequences, annealing temperature and product size for MSP assays**

Primer	Sequences(5′-3′)	Annealing temperature	Product size
SFRP1-MF	TGTAGTTTTCGGAGTTAGTGTCGCGC	62	126 bp
SFRP1-MR	CCTACGATCGAAAACGACGCGAACG		
SFRP1-UF	GTTTTGTAGTTTTTGGAGTTAGTGTTGTGT	55	135 bp
SFRP1-UR	CTCAACCTACAATCAAAAACAACACAAACA		
SFRP2-MF	GGGTCGGAGTTTTTCGGAGTTGCGC	62	138 bp
SFRP2-MR	CCGCTCTCTTCGCTAAATACGACTCG		
SFRP2-UF	TTTTGGGTTGGAGTTTTTTGGAGTTGTGT	50	145 bp
SFRP2-UR	AACCCACTCTCTTCACTAAATACAACTCA		

M, methylated; U, unmethylated; F, forward; R, reverse.

For the MSP, 3 μL of bisulfite converted DNA was used in each amplification. PCR was performed in a reaction volume of 25 μL consisting of 17.875 μL ddH2O, 2.5 μL 10 × PCR buffer,2 μL dNTP mixture,0.25 μL forward primer, 0.25 μL reverse primer, 2 μL template, and 0.125 μL TaKaRa Taq HS at the following condition: 95℃ for 5 min, followed by 40 cycles at 95℃ for 30 s, 55℃ for 30s(SFRP1-U primer), 62℃ for 30 s(SFRP1-M primer), 50℃ for 30s(SFRP2-U primer), 62℃ for 30s (SFRP2-M primer), and 72℃ for 5 min. The MSP products were then analyzed by 2.5% agarose gel electrophoresis.

Statistical analysis

To compare characteristics of the different groups of patients and biologic samples, *t* tests, χ^2 tests, and Fisher exact tests were used as appropriate. All statistical tests were two sided. Calculations were performed using SPSS10.0 software. $P < 0.05$ was considered statistically significant.

RESULTS

Hypermethylation of SFRP1 and SFRP2 gene in colorectal tissue samples

We examined hypermethylated SFRP1 and SFRP2 gene in the DNA from 39 CRCs, 34 adenomas and 17 hyperplastic polyps by MSP. SFRP1 and SFRP2 were hypermethylated in 92.3%(36/39) and 87.2%(34/39) of CRCs, in 76.5%(26/34) and 67.6%(23/34) of the adenomas and in 47.1%(8/17) and 35.3%(6/17) of the hyperplastic polyps, respectively. Notably, at least one of the two SFRP genes was hypermethylated in 94.9%, 82.4%, 52.9% of CRCs, adenomas and hyperplastic polyps. SFRP1 and SFRP2 were hypermethylated more in advanced adenomas(size ⩾ 1 cm and high-grade dysplasia) than that in the other adenomas (size < 1 cm). The prevalence of hypermethylated SFRP1 and SFRP2 were a gradually increasing tendency from hyperplastic polyps to CRCs. In contrast, none of the normal colorectal mucosa of endoscopically normal patients showed any hypermethylated bands(***Tab 2***).

Detection of hypermethylated SFRP1 and SFRP2 gene in matched fecal DNA

Following the performance of the MSP assays on the DNA extracted from the tissue samples above, we assessed fecal DNA from the same patients studied who had matched fecal and tissue samples to determine if we could detect SFRP1 and SFRP2 hypermethylation from individuals with CRCs and precancerous lesions. Hypermethylated SFRP1 was detected in 89.7%, 64.7%, 35.3% of matched fecal samples from patients with CRC, adenoma and hyperplastic polyp, respectively. Hypermethylated SFRP2 was detected in 82.1%, 55.9%, 29.4% of matched fecal samples from CRC, adenoma and hyperplastic polyp, respectively. Of these two genes, at least one hypermethylated was 94.9%, 82.4%, 52.9% in tissue samples and 92.3%, 73.5%, 47.1% in matched fecal samples from CRC, adenoma and hyperplastic polyp, respectively. CRC samples that were methylation-negative were also negative in the matched fecal DNA except for only one(sFRP-1 in patient 12; 1 of 39, 3.3%). To evaluate the clinical specificity of this assay, we next analyzed fecal DNA of 30 control individuals, all of whom were negative for colon cancer on colonoscopic exam. Only 2 samples(10.0%) tested positive for hypermethylated SFRP1 and another 1 sample (5.0%) tested positive for hypermethylated SFRP2(***Fig 1, Tab 2***). These results indicated a 92.3% sensitivity and a 85.0% specificity of the combined test of the two genes for detecting CRC and precancerous lesions.

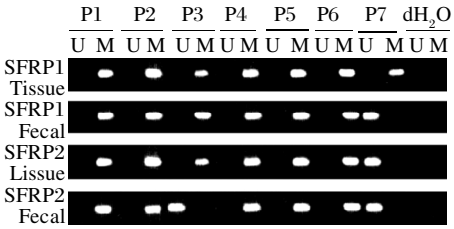

Fig 1 Hypermethylation of SFRP1 and SFRP2 gene in CRC tissues and matched fecal samples of the same patients. P, patient; M, methylated; U, unmethylated

Tab 2 **Hypermethylation of SFRP1 and SFRP2 in tissue and fecal samples**

Diagnosis	Case(n)	Tissue		Total	Fecal		Total
		SFRP1(%)	SFRP2(%)		SFRP1(%)	SFRP2(%)	
Colorectal cancer	39	36(92.3)	34(87.2)	37(94.9)	35(89.7)	32(82.1)	36(92.3)
Adenomas	34	26(76.5)	23(67.6)	28(82.4)	22(64.7)	19(55.9)	25(73.5)
Siz ⩾ 1cm	19	17(89.5)	16(84.2)	17(89.5)	14(73.7)	13(68.4)	16(84.2)
Size < 1cm	15	9(60.0)	9(60.0)	11(73.3)	8(53.3)	6(40.0)	9(60.0)
Hyperplastic polyp	17	8(47.1)	6(35.3)	9(52.9)	6(35.3)	5(29.4)	8(47.1)
Normal	20	0(0)	0(0)	0(0)	2(10.0)	1(5.0)	3(15.0)

Although the hypermethylated SFRP1 and SFRP2 gene showed a stepwise increase with pathologic grade among precancerous lesions and CRC, no significant associations were observed between the presence of methylation in fecal DNA and any clinicopathologic characteristics of CRC including sex, age, tumor stage, site and histological grade, etc. (***Tab 3***), indicating that these two hypermethylation markers in later stage CRC are as sensitive as in early stage CRC, and may be equivalently sensitive to proximal or distal cancers.

DISCUSSION

Aberrant Wnt signaling pathway is an early progression event in 90% of CRCs, contributing to the growth, proliferation and loss of apoptosis of tumor cells[16]. SFRPs are tumor suppressor proteins that contain a domain similar to one of WNT-receptor Frizzled proteins(Fz) and may block Wnt signalling either by interacting with

Tab 3 **Relation of hypermethylated SFRP1 and SFRP2 gene in fecal and clinicopathologe characteristics of CRC**

Characteristics	Case(n)	SFRP1			SFRP2		
		U	M	*P*	U	M	*P*
Sex				0.700			0.339
Male	23	2	21		3	18	
Female	16	2	14		4	14	
Age(year)				0.792			0.444
⩾50	27	3	24		4	23	
< 50	12	1	11		3	9	
TNM stage				0.370			0.062
Ⅰ / Ⅱ	21	3	18		6	15	
Ⅲ /Ⅳ	18	1	17		1	17	
Lymph node status				0.181			0.425
Positive	22	1	21		3	19	
Negative	17	3	14		4	13	
Tumor locus				0.913			0.747
Rectum	16	2	14		2	14	
Left hemicolon	10	1	9		2	8	
Right hemicolon	13	1	12		3	10	

M, methylated; U, unmethylated; $P < 0.05$ was considered statistically significant.

Wnt proteins to prevent them from binding to Fz proteins or by forming nonfunctional complexes with Fz[17]. 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[18-20]. It has been showed that hypermethylation of SFRPs genes occurs as an early event in the evolution of aberrant crypt foci(ACF)-adenoma-carcinoma sequence and is increased through carcinogenic transformation(especially hypermethylated sFRP1 and sFRP2 at human chromosome 4q31.3[21] and 8p11.21[22]) occurred frequently. SFRP1 and SFRP2 appeared to be more specific for colorectal carcinoma, adenoma and ACF, may provide potentially useful markers for predicting the risk of colorectal neoplasia[13,23,24].

Detection of tumor-derived DNA alterations in fecal samples is an intriguing new approach with high potential for the noninvasive detection of CRC[10]. 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[7-12]. However, none of them were reliable biomarkers for CRC and premalignant lesions because of their limited sensitivity/specificity. In our study, we have combination analyzed the methylation status of SFRP1 and SFRP2 in tissue and matched stool samples from patients with CRC and benign diseases. It has been shown that these two genes are frequently hypermethylated in CRC and premalignant lesions[13,18-20]. Our results showed that the clinical sensitivity of combination analysis of hypermethylated SFRP1 and SFRP2 gene in feces for detecting the presence of CRC, adenoma and hyperplastic polyp was 92.3%, 73.5% and 47.1%, respectively. Hypermethylated SFRP1 and SFRP2 occurred in hyperplastic polyps, and became more frequently in adenomas(especially advanced adenomas with size ⩾ 1cm) and CRC. Moreover, hypermethylated SFRP1 gene was found in fecal of two endoscopically normal subjects and hypermethylated SFRP2 gene was found in another endoscopically normal subjects, which may be most likely owing to the hypermethylated SFRP1 and SFRP2 occurring frequently in premaligant aberrant crypt foci(ACF) that is overlooked at colonoscopy[14,19]. These findings clearly suggested that (1) methylation detection of the SFRP1 and SFRP2 gene was feasible and reliable and (2) the combination of hypermethylated SFRP1 and SFRP2 gene in feces could be used as excellent non-invasive diagnostic biomarkers for CRC and premaligant lesions.

Detection of aberrant methylation of *SFRP* genes in fecal of CRC has been described in three studies[10]. M ü ler *et al*[25] reported that SFRP2 is methylated differentially in stools of patients with or without CRC, and SFRP2 hypermethylation is proposed as a sensitive marker, detecting 77%-90% of CRCs. Huang ZH *et al*[14,26] detected hypermethylated SFRP2 in fecal samples from Chinese patients and suggesting that hypermethylated SFRP2 occurs in 94.2%, 52.4%, 37.5%, and 16.7% of patients with CRC, adenoma, hyperplstic polyp, and ulcerative colitis, respectively. Oberwalder M *et al*[27] recently reported that SFRP2 hypermethylation in fecal DNA increases significantly from healthy controls to patients with hyperplastic polyps(33%) and to patients with adenomas(46%). All these findings suggested that SFRP2 hypermethylation may have the potential to progress to advanced adenomas, which may have a significant potential to transform into CRC. As the hyperplastic polyp and adenoma is the early event of

colorectal tumorigenesis, and has the best demonstrated risk for transformation[28,29], our results showed that hypermethylated SFRP1 and SFRP2 were detectable not only in stools of patients with CRC, but also in stools of patients with premalignant lesions such as advanced adenomas, suggesting that these two genes could be used as a panel of sensitive fecal-based biomarkers for CRC screening.

In conclusion, Multiple SFRP1 and SFRP2 gene may gradually become hypermethylated during the process of CRC development and progression. Combined analysis of hypermethylated SFRP1 and SFRP2 gene in the fecal DNA test has a high sensitivity and specificity for CRC and may be valuable for screening purposes. Compared with current CRC screening methods, stool DNA methylation test is more patient-friendly, non-invasive, more sensitive and specific. The cost-effectiveness of screening may also be improved. We believe that an optimal hypermethylation panel of SFRP1 and SFRP2 gene could significantly contribute to early detection of CRC and also the design of better antitumor interventions.

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The Genetic Epidemiology of Lung Cancer-What have we learned?

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With the completion of the Human Genome Project new opportunities have been arisen to more fully characterize the genomic factor contributing to human susceptibility to chemical and pharmacological toxicity. Over 6 million single nucleotide polymorphisms(SNPs) have been identified and cataloged in public databases. Research efforts are now underway to identify which SNPs are associated with variation in disease risk, chemical sensitivity, drug toxicity, as well as drug responsiveness.

The remarkable advances in our ability to measure the expression of thousands of genes and to genotype thousands of genetic variations has led to high expectations for the ability to discover and then apply critical new genomic information to understand human susceptibility to disease.? Similar expectations for protein and metabolic biomarkers of disease are also emerging as these technologies continue to develop.

These expectations impact several key scientific, clinical, and policy areas. First, genomic and related technologies are expected to revolutionize strategies for prediction of disease susceptibility and toxic response to environmental agents, ranging from the discovery of new biomarkers of susceptibility to the incorporation of hundreds of genomic indicators into composite toxicity markers, referred to as molecular profiles or signatures. However, as a rule, humans vary in their responses to any environmental factor because of genetic variability and epigenetic factors. Consequently, the same level of exposure to a particular chemical compound may give rise to different biological effects is different individuals. For example, irinotecan is an anticancer drug approved for combination therapy of advanced colorectal cancer. Severe life-threatening toxicities can occur in some individuals from irinotecan treatment. Although multiple genes play a role in irinotecan activity, polymorphisms in the UDP glycuronosyltransferase 1 family, polypeptide A1(UGT1A1) enzyme has been strongly associated with irinotecan toxicity. Prospective screening of patients prior to chemotherapy selection could reduce the frequency of severe toxicities by alerting physicians to the need to select alternative therapies for patients carrying the UGT1A1*28 polymorphism.

With regard to lung cancer, I will discuss and illustrate several aspects of genetic epidemiology:

1.Genetic susceptibility to lung cancer for both main heritable effects and gene-environment interactions?

2.The role of heritable factors in the outcome (survival) of lung cancer patients?

3.What is developing on the horizon?