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ORIGINAL ARTICLE

SFRP2 methylation in fecal DNA—a marker for colorectal polyps

Michael Oberwalder · Marion Zitt · Cornelia Wöntner · Heidi Fiegl · Georg Goebel · Matthias Zitt · Olivia Köhle · Gilbert Mühlmann · Dietmar Öfner · Raimund Margreiter · Hannes M. Müller

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Abstract
Introduction DNA methylation of secreted frizzled-related proteins (*SFRPs*) can be detected in colorectal cancer (CRC) tissue, in tissue of adenomas, and in aberrant crypt foci, whereas in normal colorectal mucosa tissue, *SFRP* genes are unmethylated. Recently, our study group was able to demonstrate *SFRP2* methylation as the most sensitive single DNA-based marker in stool for identification of CRC. The purpose of this study was to clarify whether *SFRP2* methylation in fecal DNA can be found in stool of individuals with hyperplastic and adenomatous colorectal polyps.
Materials and methods Patients who were diagnosed with colorectal polyps or showed negative colonoscopy were included in this study. DNA from stool samples was isolated. *SFRP2* methylation was assessed by means of MethyLight.
Results Stool samples from 68 individuals were checked for DNA content; 23% of the samples (6 of 26) from healthy controls, 46% of the samples (6 of 13) from patients with hyperplastic polyps, and 45% of the samples (13 of 29) from patients with adenomas were positive for human DNA. *SFRP2* methylation in stool samples was found in none of the healthy controls, in 33% (2 of 6) patients with hyperplastic polyps, and in 46% (6 of 13) patients with adenomas. Statistical analysis revealed that the frequency of *SFRP2* methylation increased significantly ($P=0.028$) from healthy controls to patients with hyperplastic polyps and to patients with adenomas.
Conclusions In the current study, we report for the first time that *SFRP2* methylation in fecal DNA increases significantly from healthy controls to patients with hyperplastic polyps and to patients with adenomas. *SFRP2* methylation may serve as a marker for molecular stool-based adenoma and CRC screening.

Keywords DNA methylation · *SFRP2* · Colorectal polyps · MethyLight · Fecal DNA · Stool

M. Oberwalder · M. Zitt · C. Wöntner · M. Zitt · O. Köhle · G. Mühlmann · D. Öfner · R. Margreiter · H. M. Müller (✉)
Department of General and Transplant Surgery,
Innsbruck Medical University,
6020 Innsbruck, Austria
e-mail: Hannes.Mueller@i-med.ac.at

M. Zitt · H. Fiegl · R. Margreiter
Tyrolean Cancer Research Institute,
Innrain 66,
6020 Innsbruck, Austria

G. Goebel
Department of Medical Statistics, Informatics and Health Economics, Innsbruck Medical University,
6020 Innsbruck, Austria

Introduction

Most colorectal cancers (CRC) arise from adenomatous polyps and evolve into adenocarcinomas through a stepwise histological progression sequence. Recently, it was demonstrated that CRCs may even arise from hyperplastic polyps via a serrated adenoma intermediate [1]. The time required for the development of cancer from adenoma is lengthy, with even conservative estimates indicating an interval of 5–10 years [2]. The detailed analysis of the events involved in malignant transformation led to the well-known model of the adenoma–carcinoma sequence [3–5]. Within this model, CRC is most commonly initiated by aberrant accumulation of beta-catenin in the Wingless/Wnt signaling pathway, leading to transcription of WNT target genes. However, only a small percentage of adenomas progress to carcinoma.

Although there is presently no clear way to identify which adenomas will undergo malignant transformation, transformation is associated with severe dysplasia, patient age, size of adenoma, and histological type [6]. It is now widely accepted that the transformation of normal colonic epithelium to CRC goes progressively along with accumulation of genetic and epigenetic alterations [7]. Genetic alterations present as a change in the primary DNA base sequence. Conversely, in epigenetic alterations, the primary DNA base sequence remains unchanged. Epigenetic alterations present as changes in the status of DNA cytosine methylation and histone acetylation. Methylation changes occur at a higher frequency than genetic changes do, are reversible upon treatment with pharmacological agents, and occur at defined regions in a gene [8–10].

Cytosine methylation occurs after DNA synthesis by enzymatic transfer of a methyl group from the methyl donor *S*-adenosylmethionine to the carbon-5 position of cytosine. In the mammalian genome, methylation takes place only at cytosines (C) that are located 5′ to a guanosine (G), in a C prior to G (CpG) dinucleotide. This dinucleotide is underrepresented in much of the genome, but short regions, known as CpG islands, are rich in CpG content. The genome of neoplastic cells simultaneously undergoes global genomic hypomethylation and dense hypermethylation of the CpG islands associated with gene regulatory regions. These regions are promoter regions, which are parts of a gene that are not transcribed. Hypermethylation of these regions is associated with transcriptional silencing of the associated gene, thus providing a DNA-based surrogate marker for expression status. It has been increasingly recognized over the past years that CpG islands of a large number of genes that are unmethylated in normal tissue are methylated to varying degrees in multiple types of human cancer [10], including CRC [7, 11]. Aberrant DNA methylation of specific loci has been additionally identified in the earliest precursor lesions of CRC, making DNA methylation a possible screening tool for preneoplastic and neoplastic colorectal lesions [7].

Furthermore, it has been reported that secreted frizzled-related proteins (*SFRPs*) known to be involved in Wingless/Wnt signaling are also affected by DNA methylation [12–14]. Particularly, it has been reported that hypermethylation of four genes of the *SFRP* family occurs very frequently in CRC and that a molecular marker panel of promoter hypermethylation of the *SFRP* genes may have the potential to detect virtually all CRCs [14]. Very recently, Qi et al. [13] demonstrated that methylation of *SFRP1*, *2*, *4*, and *5* can be detected not only in CRC tissue but also in tissue of adenomas and in aberrant crypt foci. In the study by Qi et al. [13], none of the normal colorectal mucosa tissue samples showed methylated bands of any of these four *SFRP* genes. Additionally, mRNA of *SFRP* genes was expressed in all normal colorectal mucosa samples, whereas mRNA expression of *SFRPs* was downregulated in carcinoma and adenoma tissue. Downregulation of *SFRP1*, *2*, *4*, and *5* expression was significantly associated with promoter hypermethylation in neoplastic colorectal tissue. Consequently, it has been postulated that hypermethylation of *SFRP* genes is a common early event in the evolution of CRC, also occurring frequently in early lesion of the multistage colorectal carcinogenesis [13].

Recently, our study group [15] was able to demonstrate *SFRP2* methylation to be the most sensitive single DNA-based marker in stool for identification of CRC (sensitivity 77–90%, specificity 77%). Furthermore, Petko et al. [16] found methylated genes in fecal DNA of individuals with colonic polyps.

The purpose of this study was to clarify whether *SFRP2* methylation in fecal DNA can be found in stool of individuals with hyperplastic and adenomatous colorectal polyps.

Materials and methods

Patients who underwent colonoscopy for various reasons and were diagnosed with benign colorectal polyps or showed negative colonoscopy were included in this study. All participants gave written informed consent and provided stool samples for DNA isolation in a self-collection approach after commencing bowel preparation the day before colonoscopy. Patients were asked to bring the stool sample with them when colonoscopy was performed. Then the stool sample was frozen at −20°C, and a copy of the endoscopy report was filed.

DNA from stool samples was isolated by means of the QIAamp DNA Stool Kit (Qiagen, Hilden, Germany). Concentration and quality of stool sample DNA was measured with a UV spectrophotometer. Sodium bisulfite conversion and DNA recovery was performed as described recently [17]. After the sodium bisulfite conversion, only samples which were positive in the MethyLight reaction for the reference gene *ACTB* were used for the further analysis. *SFRP2* methylation was assessed with MethyLight, a fluorescence-based, real-time polymerase chain reaction (PCR) assay. MethyLight is a PCR technique that is able to quantitate DNA methylation at a particular locus by using DNA oligonucleotides that anneal differentially to bisulfite-converted DNA according to the methylation status in the original genomic DNA [18]. The technique is described in detail elsewhere [19]. Nucleotide sequences for *SFRP2* and *ACTB* MethyLight primers and probes are listed in Table 1.

For statistical analysis, a one-sided likelihood ratio test was used, and calculations were performed using Statistical Package for the Social Sciences (SPSS), version 11.0, and StatXact 4 for windows.

Table 1 Sequences of used primers and probes

Gene	Forward primer sequence	Reverse primer sequence
ACTB	TGGTGATGGAGGAGGTTTAGTAAGT	AACCAATAAAACCTACTCCTCCCTTAA
SFRP2	AAACCTACCCGCCCGAAA	GTTGAACGGTGGTTGGAGATTC
Probe Oligo Sequence		
ACTB	ACCACCACCCAACACACAATAACAAACACA	
SFRP2	CGCCTCGACGAACTTCGTTTTCCCT	

Results

Stool samples from 68 individuals were included in the present study. Of those patients, 26 (mean age 60.3 yrs) had a normal endoscopic finding for the colon and rectum, 13 had hyperplastic polyps (mean age 58.7 yrs), and 29 had adenomas (mean age 58.5 yrs).

These 68 stool samples were checked for DNA content: 23% of the samples (6 of 26) from healthy controls, 46% of the samples (6 of 13) from patients with hyperplastic polyps, and 45% of the samples (13 of 29) from patients with adenomas were positive for human DNA (Fig. 1).

SFRP2 methylation in stool samples was found in none (0%) of the healthy controls, in two out of six (33%) of patients with hyperplastic polyps, and in 6 out of the 13 (46%) patients with adenomas (Table 2). Statistical analysis revealed that the frequency of *SFRP2* methylation increased significantly (P=0.028) from healthy controls to patients with hyperplastic polyps and to patients with adenomas.

Discussion

Based on current practice patterns, demand for colonoscopy exceeds supply, regardless of screening strategy. For the USA, it was estimated that colonoscopic screening every 10 years could require 32,700 more endoscopists [20]. Thus, it is reasonable to search for highly sensitive, specific, and easily analyzable stool-based screening markers for colorectal polyps and cancer [7]. Currently available genetic tests lack sensitivity and up to now cannot compete with colonoscopy due to high costs [21].

Exfoliation of DNA from colonocytes or from neoplastic colorectal cells is a continuous process and provides a steady supply of DNA. This is in contrast to bleeding from cancers and from adenomas, which occurs in episodes. This explains the relatively low sensitivity of fecal occult blood tests, lying between 15 and 30% [22]. Colonocytes shed from normal epithelium are apoptotic; these cells undergo a programmed cell death, and their DNA is digested into short fragments by endonucleases activated in the involutional process. In contrast, colonocytes shed from neoplasms appear to be non-apoptotic, and DNA integrity from neoplasm-derived cells is better-preserved [23].

Effective early detection of adenomas would offer several benefits, such as lowered CRC incidence and reduced need for surgical intervention. The optimal test would be one that accurately detected advanced adenomas with a high chance of malignant progression and that additionally detected CRC [24]. Especially genes involved

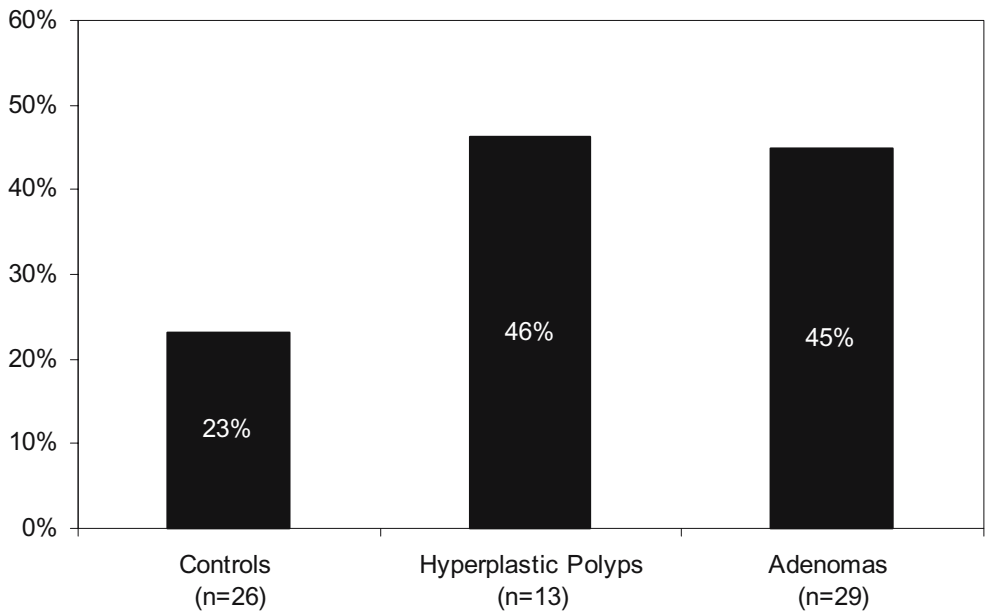

Fig. 1 Percentage of DNA-positive stool samples according to histological presentation

Table 2 Clinicopathological features of patients and controls

Patient ID	Age (years)	Sex	Col. Result	Localization	*SFRP2*
47	75	M	C	·	
89	55	F	C	·	
118	69	F	C	·	
196	62	M	C	·	
201	47	M	C	·	
301	41	M	C	·	
134	53	M	HP	LC	+
177	51	M	HP	R	
183	64	F	HP	R	+
204	46	M	HP	R	
206	57	F	HP	LC	
258	63	M	HP	R	
42	64	M	A	LC	+
191	42	M	A	LC	
52	58	F	A	ML	+
268	52	F	A	LC	
117	55	M	A	R	
95	54	F	A	UKN	+
96	36	M	A	R	+
98	50	M	A	R	
166	53	M	A	R	
192	62	M	A	LC	+
328	58	F	A	R	
330	79	F	A	LC	
362	20	M	A	ML	+

Pat. ID Patient identification number; *Col. Result*, result of colonoscopy; *F* female; *M* male; *C* control (normal mucosa); *HP* hyperplastic polyp; *A* adenoma; *R* rectum; *RC* right colon; *LC* left colon; *UKN* unknown

in Wingless/Wnt signaling, such as *SFRPs*, are believed to serve as a molecular marker of CRC [12, 14].

In a recent study, we were able to demonstrate that *SFRP2* methylation in stool represents one of the most sensitive single stool-based markers for identifying CRC, reaching a sensitivity of 77–90% and a specificity of 77% [15]. In our current study, we investigated *SFRP2* methylation in stool samples from patients with colorectal polyps, in which human DNA was detected. We found human DNA in only 23% of the samples from healthy controls and in 46 and 45% of the samples from patients with hyperplastic polyps and from patients with adenomas, respectively. A potential reason for the relatively low DNA detection rate might be found in the self-collection procedure. The samples obtained varied in the amount and consistency of stool, and some specimens were stored at room temperature for some time, allowing DNA digestion by DNAses present in the feces. Furthermore, Loktionov et al. [25] reported that greater amounts of DNA can be found in stool samples from CRC patients than from healthy controls, making greater amounts of DNA in stool samples per se an interesting test for CRC early detection and screening. Additionally, Boynton et al. [26] reported that DNA fragments isolated from stool samples from CRC patients were of higher molecular weight than were fragments isolated from fecal DNA from a colonoscopy-negative control group. Consequently, Boynton et al. [26] concluded that the presence of long DNA fragments in stool is associated with CRC and may be related to disease-associated differences in the regulation of proliferation and apoptosis.

Therefore, it seems to be plausible to find relatively low amounts of DNA in our study dealing only with patients with polyps and healthy controls. For future studies, standardized techniques will be needed for stool collection and preservation. This may increase the content of fecal DNA detected.

After checking all samples for their DNA content, we analyzed three groups of patients showing no difference in patient age to exclude possible bias due to age-dependent methylation changes, as has been reported for some methylated genes in CRC [27–29].

Finally, we detected *SFRP2* methylation in one third of the patients with hyperplastic polyps and in nearly one half of the patients with adenomas. In contrast, *SFRP2* methylation was zero in healthy controls. These findings are consistent with data recently published by Qi et al. [13], who reported that methylation of *SFRP1*, *2*, *4*, and *5* can be detected in CRC tissue, in tissue of adenomas and aberrant crypt foci, but not in normal colorectal mucosa. Furthermore, mRNA of *SFRP* genes was found to be expressed in all normal colorectal mucosa samples, whereas mRNA expression of *SFRPs* was found to be downregulated by hypermethylation in carcinoma and adenoma tissue. Additionally, Qi et al. [13] demonstrated that the epigenetic downregulation of the *SFRP* genes can be effectively restored after treatment with a combination of a demethylating agent (5-aza-2-deoxycytidin) and a histone deacetylase (TSA). This may have great impact for patients diagnosed with epigenetic molecular signs for the risk to develop cancer (e.g., *SFRP2* methylation in fecal DNA) because demethylating agents and histone deacetylases are thought to have great potential for chemoprevention [30].

Conclusion

Recently, we were able to demonstrate that methylated *SFRP2*—a gene known to be involved in Wingless/Wnt signaling—represents one of the most sensitive single stool-based markers for identifying CRC [15]. In the current study, we report for the first time that *SFRP2* methylation in fecal DNA increases significantly from healthy controls to patients with hyperplastic polyps and to patients with adenomas. *SFRP2* methylation may serve as a single fecal

molecular marker, not only for detection of CRC but also for detection of preneoplastic colorectal lesions, making it a promising candidate for molecular stool-based screening. Additionally, DNA methylation analysis may have the potential to differentiate preneoplastic lesions on a molecular level, thus improving the classification of lesions with the potential to become malignant.

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