Research letters

Methylation changes in faecal DNA: a marker for colorectal cancer screening?

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DNA methylation is a common molecular alteration in colorectal cancer cells. We report an assessment of faecal DNA from patients with colorectal cancer and controls to determine the feasibility, sensitivity, and specificity of this approach. By use of MethyLight analysis of faecal DNA from three independent sets of patients, we identified *SFRP2* methylation as a sensitive single DNA-based marker for identification of colorectal cancer in stool samples (sensitivity 90% [CI 56–100] and specificity 77% [46–95] in the training set [n=23]; sensitivity 77% [46–95] and specificity 77% [46–95] in an independent test set [n=26]). Whether a combination of genetic and epigenetic markers will identify colorectal cancer at an early stage remains to be shown.

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Colorectal cancer is among the most frequently diagnosed cancers in the industrialised world. Early detection seems to be a key factor in reducing rates of death from this disease.[1] Several methods of detection are available, including faecal occult blood test, flexible sigmoidoscopy, barium enema, and colonoscopy. However, none of these approaches has yet been established as a screening method, either because the uncomfortable and unpleasant preparation procedures are unacceptable to patients, or because of low sensitivity or specificity. Faecal DNA analysis opens up a new field for early detection of this widespread neoplasia.[2] Tagore and colleagues[3] reported a multitarget assay panel in faecal DNA analysis consisting of 21 specific mutations in the *APC*, *TP53*, and *KRAS* genes, a microsatellite instability marker (BAT-26), and a DNA integrity assay, reflecting abnormal apoptosis, with a sensitivity of 64%. In addition to these genetic alterations, changes in the status of DNA methylation, known as epigenetic alterations, count among the most common molecular alterations in human neoplasia, including colorectal cancer.[4] Moreover, it is now widely known that methylated DNA can be detected in various body fluids.

In this proof-of-principle study, we aimed to clarify whether methylation changes in faecal DNA isolated from stool samples could be used to screen for colorectal cancer. We designed a three-step prospective study, aiming to assess the most promising epigenetic markers for colorectal cancer out of a long list of candidate genes (gene evaluation set) and to test these genes in two independent sets of patients (training and test set).

DNA from stool samples was isolated by means of the QIAamp DNA Stool Kit (Qiagen, Hilden, Germany; data not shown). DNA methylation was assessed by use of MethyLight, a fluorescence-based, real-time PCR assay, as described elsewhere.[5] Patients who underwent colonoscopy for various reasons between August, 2003, and January, 2004, who gave written informed consent and provided sufficient stool samples for DNA isolation, were included in this study. The study was approved by the local institutional review board.

Initially, a gene evaluation set was used to determine the most promising epigenetic markers to identify patients with colorectal cancer. To prevent a collection bias, we used either bowel lavage fluid obtained during colonoscopy or mucus and bowel content obtained intraoperatively from nine patients with colorectal cancer and ten control patients without the disease. Next, the methylation status of 44 genes in the DNA isolated from these samples was assessed. Most of the genes assessed had been studied in serum from patients with breast cancer.[5] Additionally, four genes (*SFRP1*, *SFRP2*, *SFRP4*, and *SFRP5*) reported to be commonly methylated in colorectal cancer tissue specimens[4] and *OPCML* were assessed. The four *SFRP* genes are reported to have the potential to detect virtually all colorectal cancers, because 123 of 124 colorectal cancer tissue specimens showed methylation at one or more of these four gene loci.[4] Using three different statistical methods (Mann-Whitney *U* test using PMR [percentage of fully methylated reference] values, χ^2 contingency test, and prediction analysis for microarrays) we found ten genes showing the greatest potential for identifying colorectal cancer patients (data not shown).

Next, we obtained stool samples from patients with colorectal cancer and healthy controls undergoing colonoscopy for various reasons at the Department of General and Transplant Surgery, Innsbruck Medical University, and the Department of General Surgery, Saint Vinzenz Hospital in Zams, Tyrol. The stool was collected by the patients themselves, either the day before colonoscopy (first stool during bowel preparation) or at hospital the day before surgical intervention. Stool samples were obtained from 53 endoscopically diagnosed healthy controls, 12 patients with histologically diagnosed adenomas, 11 patients undergoing control endoscopy during colorectal cancer follow-up, and 31 patients with colorectal cancer. Before starting the DNA isolation procedure we excluded the patients diagnosed with adenomas and those having had colorectal cancer to ensure clearly defined groups of patients. Next, we determined two independent age-matched sets of patients (training and test set). Furthermore, because of the different amounts and consistency of the stool collected by the patients and possible degradation of DNA during the self-collection procedure, we checked all samples for their DNA content.

We were able to isolate DNA from 26 endoscopically diagnosed healthy controls and 23 patients with colorectal cancer (table). All people undertaking the isolation procedure and MethyLight analyses were masked to the disease status of patients. Methylation status of the ten genes identified in the gene evaluation set (*SFRP1*, *SFRP2*, *SFRP5*, *TFF1*, *PGR*, *IGFBP2*, *CALCA*, *CDH13*, *TWIST1*, *MYOD1*) was assessed in the faecal DNA of the patients (n=10) and controls (n=13), representing the predetermined training set (table). We found significant differences in DNA methylation at a specified gene locus for *SFRP2*, *SFRP5*, *PGR*, *CALCA*, and *IGFBP2* (p=0·003, 0·04, 0·03, 0·019, and 0·015, respectively; Mann-Whitney *U* test) in faecal DNA of colorectal cancer patients compared with healthy

	Indication	Colonoscopy result	Histology	Locality	Stage	PMR values				
						SFRP2	*SFRP5*	*PGR*	*CALCA*	*IGFBP2*
Patient age*, sex										
TrS/C										
63, F	ABE	NM	..	..	..	0·00	0·00	0·00	0·00	0·00
39, M	CHBH	NM	..	..	..	0·00	0·00	0·00	0·00	0·00
66, M	OTHER	NM	..	..	..	1·67	0·10	7·06	0·00	0·00
42, F	PEP	NM	..	..	..	1·84	0·06	0·36	0·00	0·00
60, M	OTHER	SP	SHP/ND	R	..	0·00	0·00	0·00	0·00	0·00
56, M	PEP	SP	SHP/ND	RC	..	0·00	0·00	0·00	0·00	0·00
44, M	PFOBT	MP	NM	R	..	0·00	0·00	0·00	0·00	0·00
69, F	CHBH	NM	..	..	..	0·00	0·18	0·00	0·00	0·00
18, M	CHBH	INFL	INFL	..	..	0·00	0·07	0·00	0·00	0·00
46, F	CHBH	DIV	NM	..	..	0·00	0·00	0·00	0·00	0·00
67, F	CHBH	MP	MHP/ND	R	..	367·53	8·19	10·77	0·00	0·00
32, M	CHBH	MP	MHP/ND	R	..	0·00	0·09	0·00	0·00	0·00
46, F	OTHER	INFL	NM	..	..	0·00	0·00	0·58	nd	0·00
TrS/CRC										
80, M	UNKN.	CA	AC	LC	pT2	37·05	4·16	12·15	0·00	0·00
65, M	CHBH	CA	AC	R	pT3	0·09	0·06	0·07	0·00	0·00
46, M	ABE	CA	AC	RC	pT3	5·28	0·28	0·47	0·03	0·00
85, F	UNKN	CA	AC	RC	pT4	2·41	0·16	0·73	3·47	0·00
68, F	CA	CA	AC	R	pT3	87·22	3·45	0·00	0·00	0·24
84, M	CA	CA	AC	R	pT3	2·67	2·06	1·01	2·15	0·00
67, M	CA	CA	AC	LC	pT3	1·88	1·51	0·73	0·25	0·07
57, F	UNKN	CA	AC	LC	pT2	5·77	0·05	12·38	0·00	0·00
55, M	ABE	CA	AC	LC	pT3	0·00	0·00	0·00	0·00	0·00
74, M	PFOBT	CA	AC	R	pT3	4·51	nd	2·73	0·00	0·00
TS/C										
40, F	CHBH	NM	..	..	..	0·00	0·00	0·00	0·00	0·00
40, M	CHBH	SP	SHP/ND	LC	..	0·00	0·00	19·85	0·00	0·00
36, F	CHBH	DIV	..	..	..	0·00	0·00	0·00	0·00	0·00
65, F	CHBH	NM	..	..	..	0·00	0·00	0·00	0·00	0·00
48, M	ABE	SP	SHP/ND	LC	..	0·00	0·81	0·09	0·00	0·00
47, M	OTHER	MP	MHP/ND	LC	..	19·19	0·00	0·51	0·00	8·09
58, M	ABE	NM	..	..	..	7·02	0·02	0·29	0·00	0·00
54, F	PFOBT	NM	..	..	..	0·00	0·00	0·00	0·00	0·00
47, F	UNKN	SP	INFL	LC	..	0·00	0·00	0·00	0·00	0·00
26, M	CHBH	NM	NM	..	..	0·00	0·00	0·00	0·00	0·00
42, M	OTHER	DIV	NM	..	..	0·00	3·46	0·00	0·00	0·00
74, F	OTHER	DIV	..	..	..	31·28	0·00	0·00	0·00	0·00
63, M	OTHER	MP	MHP/ND	LC	..	0·00	0·00	0·00	0·00	0·00
TS/CRC										
84, M	UNKN	CA	AC	LC	pT3	8·85	0·21	7·10	0·32	0·00
64, F	UNKN	CA	AC	LC	pT3	0·00	0·00	1·89	0·00	0·00
63, M	UNKN	CA	AC	R	pT3	3·39	1·09	0·90	2·49	0·00
90, M	PFOBT	CA	UNKN	R	pT3	3·81	2·47	2·26	0·61	0·00
34, F	UNKN	CA	AC	R	pT3	0·00	0·00	0·00	0·00	0·00
44, M	CA	CA	AC	RC	pT3	2·00	1·03	0·64	0·00	0·00
63, M	CA	CA	AC	LC	pT4	39·44	75·64	24·98	15·10	0·00
56, M	CA	CA	AC	LC	pT3	28·33	5·33	2·56	0·00	0·02
59, M	CA	CA	AC	R	pT3	9·00	156·95	0·00	0·00	0·00
62, M	CA	CA	AC	R	pT3	14·48	0·78	0·65	0·00	0·00
81, F	CA	CA	AC	LC	pT2	38·76	0·22	3·98	0·00	0·00
68, M	CA	CA	AC	RC	pT2	0·00	0·00	0·00	0·00	0·00
69, F	UNKN	CA	AC	LC	pT3	9·88	10·59	0·34	0·00	0·00

TrS/C=training set, controls. TrS/CRC=training set, patients with colorectal cancer. TS/C=test set, controls. TS/CRC=test set, patients with colorectal cancer. F=female. M=male. ABE=abnormal barium enema. CHBH=changes in bowel habits. OTHER=other reasons. PEP=pre-existing polyp. PFOBT=positive faecal occult blood test. UNKN=unknown. CA=carcinoma. CRC=colorectal cancer. NM=normal mucosa. SP=single polyp. MP=multiple polyps. INFL=inflammation. DIV=diverticulosis. SHP/ND=single hyperplastic polyp without dysplasia. MHP/ND=multiple hyperplastic polyps without dysplasia. AC=adenocarcinoma. nd=not determined. R=rectum. RC=right colon. LC=left colon. *Years.

Clinicopathological features of patients

controls; nine of ten patients with colorectal cancer and three of 13 without the disease had methylated *SFRP2* in their faecal DNA (sensitivity 90% [CI 56–100] and specificity 77% [46–95]).

These findings were then assessed in the faecal DNA of an independent test set (13 patients with colorectal cancer and 13 controls; table). Analysis using PMR values showed that three genes were still differentially methylated between patients with and without colorectal cancer (p=0·017, 0·017, and 0·047 for *SFRP2*, *SFRP5*, and *PGR*, respectively; Mann-Whitney *U* test); *SFRP2* was shown to be methylated in the faecal DNA in ten of 13 patients with colorectal cancer and three of 13 without the disease (sensitivity 77% [CI 46–95] and specificity 77% [46–95]).

The findings of this proof-of-principle study show that a sole DNA-based marker (*SFRP2*), assessed in independent sets of patients, has a sensitivity of 77–90% for identifying patients with colorectal cancer. Since most of our control patients were symptomatic due to various diseases (table), the specificity of 77% might be higher when asymptomatic healthy controls are investigated. We established this self-collection approach as an easy procedure for patients in order to increase their willingness to participate in this screening test, which besides sensitivity and specificity is an important criterion for introducing a screening test into clinical routine. We are aware that this self-collection approach might represent a possible shortcoming, which should be addressed in further studies by more standardised collection procedures.

Nevertheless, to our knowledge *SFRP2* methylation represents one of the most sensitive markers for identifying colorectal cancer, besides mutation analysis[3] and protein analysis,[6] in stool samples. Whether a panel of genetic and epigenetic markers in stool could be used to identify colorectal cancer at an early stage remains to be shown.

Contributors

Experimental work was done by H Fiegl, and M Morandell. H M Müller, M Oberwalder, M Zitt, M Mühlthaler, and D Öfner recruited patients and collected samples. All authors were involved in data analysis. Statistical analysis and study design were done by G Goebel. R Margreiter had overall responsibility for patient care. H M Müller and M Widschwendter designed the study, did data analysis and interpretation, and wrote the manuscript. M Widschwendter initiated and supervised the entire study. H M Müller, M Oberwalder, and H Fiegl contributed equally to this work.

Conflict of interest statement

None declared.

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Isolation of *Salmonella enterica* serotype choleraesuis resistant to ceftriaxone and ciprofloxacin

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***Salmonella enterica* serotype choleraesuis (*S choleraesuis*) usually causes systemic infections in man that need antimicrobial treatment. We isolated a strain of *S choleraesuis* that was resistant to ceftriaxone and ciprofloxacin from a patient with sepsis. Ciprofloxacin resistance was associated with mutations in *gyrA* and *parC*, whereas the *ampC* gene (bla_{CMY-2}), responsible for ceftriaxone resistance, was carried by a transposon-like mobile element. This element was found inserted into *finQ* of a potentially transmissible 140 kb plasmid, with an 8 bp direct repeat flanking the junction regions. The appearance of this resistant *S choleraesuis* is a serious threat to public health, and thus constant surveillance is warranted.**

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Salmonella enterica serotype choleraesuis (*S choleraesuis*) usually causes systemic infections in man that need antimicrobial treatment. The emergence of *S choleraesuis* that is resistant to multiple antimicrobial agents—notably, fluoroquinolones—has aroused concern about the treatment of systemic non-typhoidal salmonellosis.[1] Nevertheless, ceftriaxone-resistant *S choleraesuis* has not, until now, been a clinical problem. We report a resistant *S choleraesuis* strain in a patient with sepsis, and our investigation into the genetic basis of that resistance.

In January, 2002, a 58-year-old man with a history of oesophageal cancer was admitted to hospital because of sepsis. From his blood culture, a strain of *S choleraesuis* was isolated and proved resistant to all antimicrobial agents commonly used to treat salmonellosis, including ciprofloxacin-ceftriaxone (table). Treatment with imipenem-cilastatin was initiated, but the patient died 7 days after admission.

We examined the *S choleraesuis* isolate SCB67 from this patient, and another strain SCB43[1] from an unrelated patient with sepsis. We obtained signed consent from the family of the patient, and the research was approved by the Chang Gung ethics committee. We identified salmonella isolates using standard methods: biochemical reactions and agglutination tests with specific antisera (Difco Laboratories, Detroit, MI, USA). The isolates were serogroup C1 salmonella unable to use citrate as a sole carbon source. H antiserum further confirmed the isolates to be *S choleraesuis* in tube-agglutination tests. An *S typhi* strain, NCTC8393, was the control.

We used a standard broth-microdilution method to check the susceptibility of the isolates, and interpreted the results according to the criteria of the National Committee for Clinical Laboratory Standards. The minimum inhibitory concentrations of ceftriaxone and ciprofloxacin against SCB67 were both at a resistant value of 16 μg/mL. Furthermore, a confirmatory test recommended by the committee showed that the minimum inhibitory concentrations of cefotaxime (16 μg/mL) and ceftazidime (32 μg/mL) to SCB67 remained unchanged irrespective of the presence or absence of clavulanic acid, and that of cefoxitin was at a resistant value (64 μg/mL). These findings suggest that SCB67 might produce AmpC but not extended-spectrum β lactamases.

PCR assays showed that the 50 kb plasmids of SCB67 and SCB43 were both *spvC*-positive, indicating that they were the serotype-specific virulence plasmid of *S choleraesuis*.[1] To detect the *ampC* gene, we next did PCR and sequencing as described earlier,[2] and amplified a 1143 bp fragment with complete homology to the bla_{CMY-2} gene from SCB67, but not from SCB43 or the control (table). *ampC* is a group of resistance genes that includes bla_{CMY-2}. Southern-blot hybridisation with the PCR product as the probe confirmed that bla_{CMY-2} was located on the 140 kb plasmid of SCB67. This plasmid was non-conjugative, but it did contain an *oriT*

	Ceftriaxone resistance gene	Mutations in DNA gyrase and topoisomerase genes	Plasmid size (kb)	Antibiotic resistance phenotype*
NCTC8393	None	None	None	T
SCB43	None	*gyrA*, *parC*	90, 50	ACSSuTGmKTpCip
SCB67	bla_{CMY-2}	*gyrA*, *parC*	140, 50	ACSSuTGmKTpCroCip

*A=ampicillin. C=chloramphenicol. S=streptomycin. Su=sulfonamide. T=tetracycline. Gm=gentamicin. K=kanamycin. Tp=trimethoprim. Cro=ceftriaxone. Cip=ciprofloxacin.

Antimicrobial susceptibility, plasmid profile, and antimicrobial resistance phenotype of the clinical isolates of *S choleraesuis* (SCB43 and SCB67) and the control strain (NCTC8393)