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Non-invasive detection of colorectal tumours by the combined application of molecular diagnosis and the faecal occult blood test

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Abstract

The treatment of early-stage tumours decreases the overall mortality of colorectal tumour patients. In this retrospective study we determined the sensitivity and the specificity of the faecal occult blood test (FOBT) and the molecular diagnosis (MD). We analysed 57 stool samples from patients with colorectal carcinomas for the presence of occult blood using a standard FOBT and for alterations in the three different tumour relevant markers APC, BAT26 and L-DNA. Stool samples from 44 control donors were analysed to determine the specificity of the applied methods. Twenty-nine (51%; 95% confidence interval (CI): 38–63%) stool samples of the cancer patients gave positive FOBT results. Thirty-seven (65%; CI: 52–76%) samples showed alterations in at least one DNA marker. Sixteen (28%) samples were positive only in the FOBT, and 24 (42%) samples showed a positive result exclusively in MD. The combined application of both methods resulted in a sensitivity of 93% (CI: 83–97%) and an overall specificity of 89% (CI: 76–95%). The combined application of FOBT and MD resulted in an overall sensitivity, which could not be achieved by any of the methods alone and which is in the range of invasive diagnostic methods.

Keywords: Non-invasive colorectal cancer diagnosis; Faecal DNA analysis; Molecular diagnosis; Faecal occult blood test

1. Introduction

The diagnosis and the therapy of early-stage tumours have the potential to decrease morbidity and mortality of colorectal cancer patients. Invasive methods like sigmoidoscopy, colonoscopy and

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roentgenography are valuable tools for the specific and sensitive detection and the exact localization of a colorectal tumour (reviewed in [1]). These techniques are unsuitable for broad screening programs mainly because of their low general acceptance [2]. The most common screening method is the faecal occult blood test (FOBT). Randomised trials revealed the clinical effectiveness in reduction of mortality through the implementation of the FOBT [3–5].

Sidransky et al. introduced the analysis of faecal DNA for tumour specific alterations as a novel non-invasive diagnostic strategy [6]. Until now several studies have proven the usefulness and the feasibility of faecal DNA analysis (reviewed in [7]). Recent multi-target studies achieved sensitivities between 62 and 91% and specificities up to 98%, respectively [8–10]. A few studies compared the outcomes of the molecular diagnosis (MD), which is the detection of tumour relevant alterations in faecal DNA, with these of the FOBT. The finding that 9 out of 11 asymptomatic adenoma patients were identified by the analysis of five different DNA markers, whereas none was detected by FOBT, indicated a higher diagnostic sensitivity of the MD [9]. The data of a large prospective study, which is close to completion, will allow assessing the two methods and their combined application (annual meetings of the American College of Gastroenterology (ACG), Baltimore 2003 and of the American Gastroenterology Association (AGA), New Orleans 2004). We intended to judge in this retrospective study whether the analysis of three faecal DNA markers has the potential to complement or even to replace the FOBT and whether the combined application of the two methods might increase the overall diagnostic reliability.

2. Materials and methods

2.1. Study population and sample collection

All patients and control donors provided their informed oral and written consent to provide samples. This study was approved by the responsible ethical committees at the Klinik für Chirurgie Marienhospital (Herne) and at the Berufsgenossenschaftliche Kliniken Bergmannsheil (Bochum). None of the patients and none of the control donors had familial adenomatous polyposis coli or hereditary non-polyposis colorectal cancer. Patients were diagnosed with colorectal tumours at the Berufsgenossenschaftliche Kliniken Bergmannsheil, the Marienhospital Herne or the Katholisches Krankenhaus Dortmund-West between 1998 and 2003. All tumours were diagnosed and located by imaging methods like colonoscopy and roentgenography. At least seven days after colonoscopy and between 2 and 4 days before surgical removal of the tumour 2 samples were taken from regular stools, immediately frozen at −20 °C and transferred to −80 °C within 24 h. The surgically removed tumours were classified according to the AJCC/TNM system (American Joint Committee on Cancer (AJCC) stages I through IV; Table 1) [11]. Because of personnel alternations in the clinical departments in the course of the study, we could not reproduce the staging information for 12 tumours, the age and the gender of 8 patients and the location for 3 tumours at the time of the analysis of the faecal samples. Control stool samples were from 24 female and 20 male donors, who volunteered to participate. The age group of control donors was 24–78 years (mean age 49). Control donors did not show any symptoms or signs of colorectal diseases as determined in personal interviews.

2.2. FOBT

Two stool samples from each cancer patient and one sample from each control donor were analysed for the presence of occult blood by a standard guaiac test using commercial reagents and standard protocols (Care Diagnostica). Each FOBT was performed twice. A blue colour reaction within 120 s after the addition of the hydrogen peroxide solution was rated as a positive result. In case the double FOBT analysis of one stool sample or of two stool samples from one patient gave discrepant results, the sample was rated positive.

2.3. MD

Faecal DNA from all samples was purified using a method, which was optimised for faecal specimen [12]. An essential step of this method is the matrix-based elimination of PCR inhibiting and DNA damaging compounds (Stool Mini Kit, Qiagen).

Table 1
Patient and tumor data of all samples and results obtained by the analysis of stool samples from cancer patients by FOBT and by MD

Patient	Gender/age	AJCC/TNM	FOBT	MD positive markers
79	M/70	n.a.*	neg	neg
80	M/58	II/T3N0MxG2	pos	APC
83	M/67	II/T3N0MxG2	neg	APC, L-DNA
84	M/85	II/T3N0MxG2	neg	BAT26
87	n.a.	n.a.	neg	APC
88	F/76	II/T3N0MxG2	pos	neg
89	n.a.	n.a.	neg	L-DNA
90	F/62	I/T2N0MxG2	neg	L-DNA
92	F/53	n.a.	pos	neg
93	n.a.	n.a.	pos	APC
95	F/68	I/T2N0MxG2	pos	APC
96	n.a.	n.a.	neg	APC, L-DNA
121	M/51	I/T2N0MxG2	neg	L-DNA
136	M/61	I/T2N0MxG2	neg	APC
138	M/65	I/T2N0MxG2	pos	neg
139	M/74	II/T3N0MxG2	neg	neg
140	F/73	II/T3N0MxG2	pos	neg
141	n.a.	n.a.	pos	APC
172	F/75	II/T3N0MxG2	neg	APC
173	M/73	II/T3N0MxG2	neg	APC
174	F/79	IV/T3N2M1hepG2	pos	APC, L-DNA
175	F/75	II/T3N0MxG3	pos	neg
176	M/63	IV/T3N1M1hepG3	neg	APC
179	M/68	III/T3N1MxG2	neg	APC
180	F/65	II/T4N0MxG2	neg	APC
181	F/92	III/T4N1M0G2	pos	neg
182	M/74	I/T2N0MxG2	neg	APC
183	M/60	II/T3N0MxG2	neg	L-DNA
184	M/65	IV/T3N2M1hepG2	neg	L-DNA
185	M/75	I/T2N0MxG2	pos	BAT26
186	M/47	III/T3N1MxG2	neg	L-DNA
187	M/73	II/T3N0MxG2	pos	neg
188	M/65	IV/T3N1M1hepG2	neg	neg
189	M/76	III/T3N2MxG2	neg	APC, L-DNA
190	M/69	I/T2N0MxG2	pos	BAT26, L-DNA
191	M/76	III/T3N1MxG2	pos	L-DNA
192	M/89	II/T3N0MxG2	pos	L-DNA
193	F/64	I/T2N0MxG2	neg	BAT26, L-DNA
251	M/55	IV/T3N2M1G2	pos	L-DNA
252	M/74	II/T3N0MxG2	pos	neg
253	F/83	III/T3N1MxG2	neg	L-DNA
254	M/66	II/T4N0MxG2	pos	BAT26, L-DNA
255	M/62	III/T3N2MxG2	pos	neg
257	n.a.	n.a.	neg	APC
274	F/70	I/T2N0MxG3	pos	neg
275	n.a.	n.a.	pos	APC, L-DNA
276	n.a.	n.a.	neg	L-DNA
278	M/86	n.a.	neg	L-DNA
283	M/82	III/T3N1MxG2	pos	APC, L-DNA
284	F/72	n.a.	neg	L-DNA
285	M/81	II/T3NxMxG2	neg	neg
286	M/64	IV/T3NxM1hepG2	pos	neg

(*continued on next page*)

Table 1 (*continued*)

Patient	Gender/age	AJCC/TNM	FOBT	MD positive markers
287	M/92	III/T3N1MxG3	pos	neg
289	M/71	II/T4NxMxG3	pos	neg
290	M/66	IV/T4N0M1	pos	neg
292	M/80	I/T1N0MxG2R0	pos	neg
294	F/83	III/T3N2MxG2	pos	neg

n.a., data not available

The quality of the purified DNA was validated by agarose gel electrophoresis. One tenth of the DNA obtained from one stool sample was used in a capturing step to enrich the human DNA from the DNA of non-human origin [13]. The overall yield of the capturing could be increased by the restriction digestion of the DNA with the enzyme DraI. To fish the APC gene out of the pool of genomic DNA, 10 pmol biotin labelled primer APC1 (5′-TTAAAA-TATGCCACAGATATTCCT TCA TCACAGAAA-CAGT-3′) was added to 100 μl DraI digested stool DNA. After denaturing and re-annealing 10 μl streptavidin coupled magnetic beads (Dynal) were added and the mixture was incubated over night at room temperature. After washing, the beads coupled DNA was completely used as template in a first PCR. The APC gene region from codon 1069 through codon 1984 including the mutation cluster region was analysed by PCR and by PTT of two overlapping fragments. The detailed experimental conditions of the following PTT have been published [13] (Hauss and Müller, The protein truncation test in mutation detection and molecular diagnosis, in: Methods in Molecular Biology, edited by Guido Grandi, The Humana Press, in press). Briefly, the primers of the first PCR coded the HA-tag, and the primers of the second PCR coded the Kozak motif and the T7 promoter sequence, which are necessary for the following PTT. The first PCR was carried out at the following conditions: 95 °C (5 min)//[94 °C (30 s)/56 °C (30 s)/72 °C (90 s)] 20×//[94 °C (30 s)/58 °C (30 s)/72 °C (90 s)] 20×//72 °C (10 min) and under the use of the following primers: APC fragment I: 5′-CGCCATGTACCCCTACGACGTGCCCGACTAC GCCTTCCAACCACATTTTGGACAGCAG-3′ and 5′-CCGTCATTTTTCTGCCTCTTTCTCTTGGTT-3′; APC fragment II: 5′-CGCCATGTACCCCTAC-GACGTGCCCGACTACGCCGATGTGGAATTAA-GAATAATGCCT-3′ and 5′-CCGTCATTTTCTT-TATTGTTGTTTTCTTGGTC-3′. One fifth of the volume of the first PCR was used in a second PCR at the conditions: 95 °C (5 min)//[94 °C (30 s)/60 °C (30 s)/72 °C (90 s)] 40×//72 °C (10 min) and using the following primers, which anneal within the products of the first PCR: APC fragment I: 5′-ATCCTAATACGACTCACTATAGGGAGCCAC-CATGTACCCCTACGACGTG-3′ and 5′-CCGTCA-TTTTTCTGCCTCTTTCTCTTGGTT-3′; APC fragment II: 5′-ATCCTAATACGACTCACTATAGGG-AGCCACCATGTACCCCTACGACGTG-3′ and 5′-CCGTCATTTTCTTTATTGTTGTTTTCTTGGTC-3′. Three of the control samples gave no reproducible results in the PCR reactions. All other faecal DNA samples from control donors and all samples from cancer patients gave robust PCR products. DNA purified from two stool samples from each cancer patient and from one sample from each control donor was analysed for the presence of alterations in 3 different markers. Each positive result was confirmed in a repeated analysis. All samples showing signals, which were different to the wild-type controls, were further examined. For this the PCR fragments were cloned in a T/A cloning vector and the cloned gene fragments were sequenced by conventional methods. The analyses of all positive samples were repeated by starting with the purified DNA in order to limit the number of false positive results. The analysis of alterations in the microsatellite marker BAT26 and the detection of the relative amount of non-apoptotic L-DNA were performed according to published protocols [9,14]. All results are based on the analysis of at least two independently amplified PCR products. The primer sequences for L-DNA analysis were kindly provided by A.P. Shuber and D.A. Ahlquist. In case of discrepant MD results between the two different samples from one patient, DNA was extracted from a new faecal aliquot of the same patient and the analysis was repeated.

Table 2
Localization of tumours relative to the splenic flexure of the colon and number of the corresponding stool samples diagnosed as positive by FOBT or by MD

Site	*N*	FOBT	MD	Combined use
Proximal	27	16	14	25
Distal	27	11	21	26
n.a.	3	2	2	2
Total	57	29	37	53

N, number of samples; n.a., at the time of analysis the information of the tumour localization was not available.

2.4. Statistical evaluation

Ninety-five percent confidence intervals were determined based on the exact binomial distribution. The Fisher exact test was used for comparison of the test results between patients with different tumour stages. To compare the single application of FOBT or MD with the combined application of both methods the McNemar's test was used.

3. Results

The aim of this study was to determine the sensitivity and the specificity of the two non-invasive diagnostic methods, FOBT and MD, which is the analysis of faecal DNA for alterations in the three different markers APC, BAT26 and L-DNA. We analysed stool samples from 57 colorectal cancer patients and from 44 control donors. Twenty-seven tumours were located proximal and 27 tumours were located distal to the splenic flexure of the colon (Table 2). Twenty-eight patients had tumours, which did not spread to proximal or distant lymph nodes (stage I or II) at the time of staging diagnosis. Lymphatic spread was identified in 17 patients (stage III or IV) (Tables 1 and 3).

Twenty-nine stool samples (51%; 95% confidence interval (CI): 38–63%) showed a positive FOBT test (FOBT pos). Thirty-seven faecal DNA samples (65%; CI: 52–76%) were MD positive (MD pos) demonstrating an alteration in at least one of the three tested DNA markers (Tables 1 and 3). The MD positives include nineteen samples (33%) with truncated APC protein fragments in the PTT analysis (Fig. 1). We sequenced these positive samples and found mutations, which result in stop codons between codons 1254 and 1472 (Table 4). The sizes of the predicted translation products of PCR fragments carrying these stop codons correlate with the approximate sizes of the translation products, which were detected in the PTT (Fig. 1). The number of APC mutations detected in the samples was in the range of the numbers

Table 3
Summarized results of the analysis of the stool samples from cancer patients and sensitivity of MD and FOBT

AJCC stage	n.a.	I	II	III	IV	Total
N	12	11	17	10	7	57
Mean age (range)	70.3 (53–86)	67.2 (51–80)	72.7 (47–92)	76.1 (47–92)	65.3 (55–79)	70.9 (47–92)
Gender (male/female)	2/2	7/4	12/5	7/3	6/1	34/15
n.a.	8	0	0	0	0	8
FOBT pos	4	6	9	6	4	29 (51%; 38–63%)
MD pos	10	8	9	6	4	37 (65%; 52–76%)
APC	6	3	5	3	2	19 (33%)
BAT26	0	3	2	0	0	5 (9%)
L-DNA	6	4	4	5	3	22 (39%)
MD pos or FOBT pos	11	11	15	10	6	53 (93%; 83–97%)
MD(APC or L-DNA) pos or FOBT pos	11	11	14	10	6	52 (91%; 81–96%)
MD pos and FOBT neg	7	5	6	4	2	24 (42%; 30–55%)
FOBT pos and MD neg	1	3	6	4	2	16 (28%; 18–41%)

The results, the relative sensitivities and the corresponding 95% confidence intervals are given for the FOBT, the MD and the combined application of the two methods. A sample was rated MD positive (MD pos), when one molecular marker was tested positive in the PTT, in the BAT26 or in the L-DNA analysis. n.a., AJCC staging of the corresponding tumours or age and gender were not available at the time of analysis.

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